

1988

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Christopher Henry Kennedy

Louisiana State University and Agricultural & Mechanical College

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**A study of the interaction of peroxidic tumor promoters with
mitochondria**

Kennedy, Christopher Henry, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1988

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A Study of the Interaction of Peroxidic
Tumor Promoters With Mitochondria

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
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in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Chemistry

by

Christopher Henry Kennedy
B.S., Edinboro University of Pennsylvania, 1983
December 1988

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I would like to dedicate this dissertation to my wife Laura and to my father and mother, John and Patricia Kennedy. Without their love and support, I would not have accomplished this important goal in my life.

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FOREWORD

This dissertation is divided into four chapters. The first chapter is a general introduction that presents background material on the functioning of mitochondria and techniques used in mitochondrial research. It also describes the physiological significance of the peroxidic compounds studied in the following chapters.

Chapter two is a manuscript entitled "Benzoyl Peroxide Interaction With Mitochondria: Inhibition of Respiration and Induction of Rapid, Large-Amplitude Swelling." This paper has been submitted for publication to Archives of Biochemistry and Biophysics. This work was presented as a poster at the 194th National Meeting of the American Chemical Society in September 1987. An abstract of this presentation was published in Biochemistry, Vol. 26, p. 4169 (1987). It will also be presented as a poster at the Gordon Conference on Oxygen Radicals in February 1989 by Dr. Pryor.

Chapter three is a communication entitled "Hydroperoxide-Induced Radical Production in Liver Mitochondria" that was published in Biochemical and Biophysical Research Communications, Vol. 141, pp. 1123-1129.

Chapter four is a manuscript also entitled "Hydroperoxide-Induced Radical Production in Liver Mitochondria." This paper is a continuation of the work

described in chapter three. It is currently in preparation and will be submitted to Archives of Biochemistry and Biophysics in the near future. It will also be presented as a poster at the Gordon Conference on Oxygen Radicals in February 1989 by Dr. Church.

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ABSTRACT

When micromolar concentrations of benzoyl peroxide (BPO) are added to rat liver mitochondria, inhibition of mitochondrial NADH-oxidase and succinoxidase is observed. The addition of a classical uncoupler of oxidative phosphorylation results in only partial release of this inhibition, suggesting that BPO inhibits both electron- and energy-transfer in mitochondria. Difference spectroscopy and artificial electron donor results indicate that BPO interacts at coupling site II between cytochromes b and c₁. BPO only yields radicals in mitochondria via inhibition of electron transport, which results in the formation of superoxide radicals. BPO also induces rapid, large-amplitude swelling of mitochondria; the swelling is dependent on the presence of monovalent cations but is independent of the presence of calcium, oxygen and respiratory substrate. BPO-induced swelling appears to be disassociated from radical production and lipid peroxidation.

When isolated rat liver mitochondria are treated with millimolar concentrations of tert-butyl hydroperoxide (TBHP), methyl, tert-butoxyl and tert-butylperoxyl radicals are detected using the ESR spin-trapping technique. The addition of respiratory substrate results in a significant increase in methyl radical production, no change in the concentration of tert-butoxyl radicals, and complete loss

of tert-butylperoxyl radicals. One-electron reduction of TBHP by the electron transport chain results in the formation of tert-butoxyl radicals, which subsequently undergo beta-scission to form methyl radicals. The site of interception of electrons by TBHP is shown to be cytochrome c_1 by the use of classical chain inhibitors and depletion of mitochondrial cytochrome c . TBHP also appears to interact with a tightly-liganded metal in non-respiring mitochondria, as metal chelators have no effect on TBHP-induced radical production while sodium cyanide inhibits this process. TBHP induces lipid peroxidation in both respiring- and non-respiring mitochondria, as measured by the thiobarbituric acid test, although the degree of peroxidation is less in respiring mitochondria. Radical production is also observed when either cumene hydroperoxide or linoleic acid hydroperoxide is added to mitochondria in the presence of spin trap. The mechanisms of TBHP-induced radical production in both respiring- and non-respiring mitochondria are discussed.

CHAPTER ONE

INTRODUCTION TO MITOCHONDRIAL RESEARCH

Morphology of Mitochondria. The average mitochondrion is an oval particle, 1 - 2 μm long and 0.5 - 1 μm wide; these dimensions are approximately the same in all sources of this organelle (1).

Mitochondria are composed of two different membranes (Fig. 1). The outer membrane is a continuous bag that encloses the entire contents of the mitochondrion. The inner membrane has a larger surface area than the outer membrane, resulting in a series of folds that project into the interior space; these folds are referred to as mitochondrial cristae (2).

Two internal compartments are found in the mitochondrion (Fig. 1). The intermembrane (or intracristal) space is located between the outer and inner membranes, and the matrix (or intercristal space) is enclosed by the inner membrane (2). The relative areas of these two spaces are dependent on the conformation of the mitochondria. The conformation is, in turn, contingent on the physiological state of the mitochondria (3). During a state of low energy (state 4 respiration, ADP is the limiting factor), the mitochondrion assumes an orthodox conformation in which the cristae are folded into sheets and the matrix occupies most of the internal space (Fig.

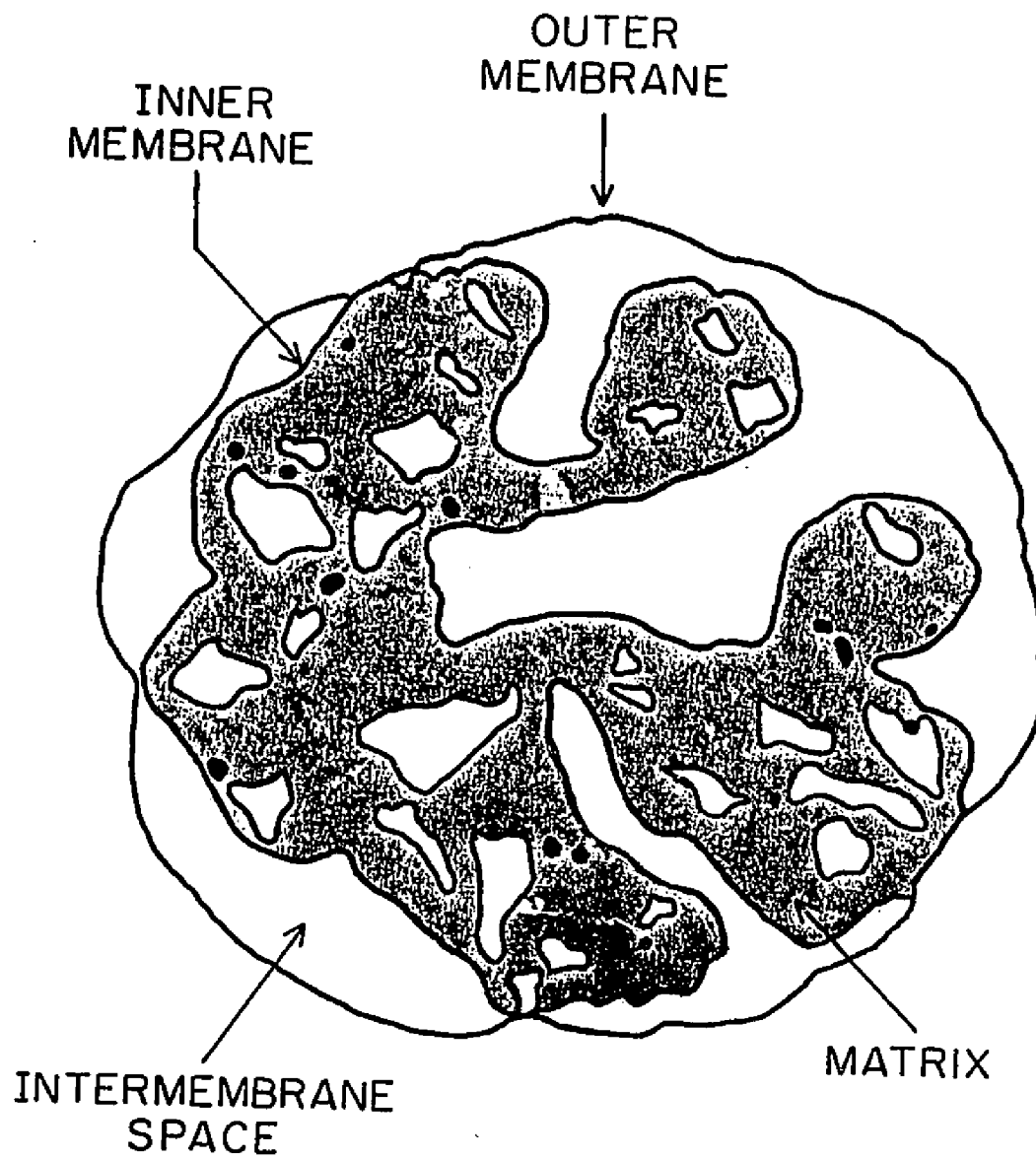


Figure 1: Morphology of the mitochondrion.

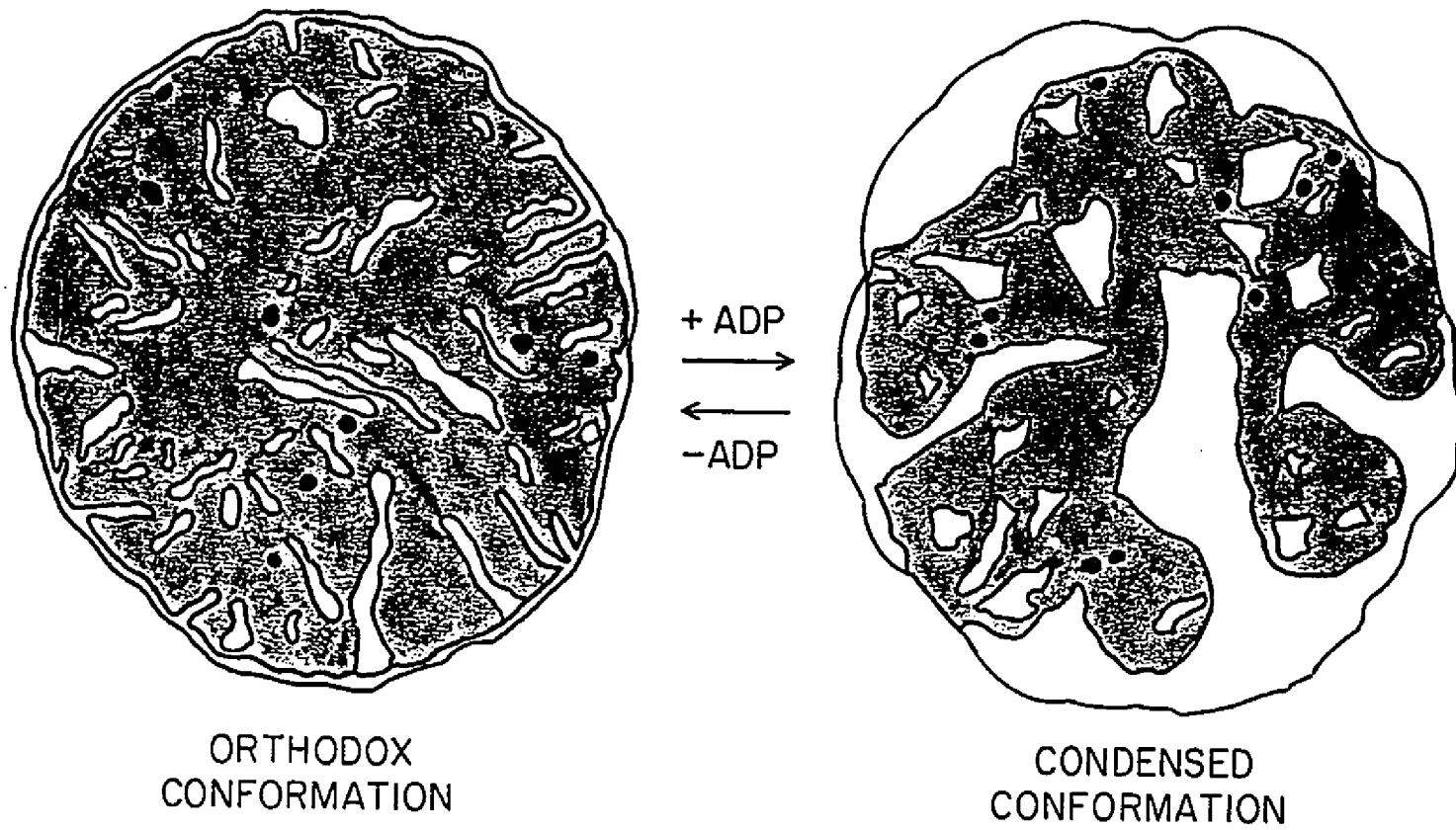


Figure 2: Energy-linked conformational changes in the mitochondrion.

2). In a state of high energy (state 3 respiration, rate of electron transfer is the limiting factor), the spatial arrangement of the inner membrane changes to a condensed conformation in which the intermembrane space increases because of a separation of the cristae surfaces; this results in a decrease in the volume of the matrix (Fig. 2). These energy-linked changes in conformation provide a rationale for the observation of low-amplitude mitochondrial swelling when this organelle undergoes a change from state 3 to state 4 respiration (4). Since energy-linked structural changes are reversible (3), low-amplitude swelling is also a reversible process.

Unlike low-amplitude swelling, large-amplitude mitochondrial swelling is not necessarily related to the energy state of the mitochondrion. Exposure of mitochondria to either an isotonic medium containing permeable cations and anions or a hypotonic medium results in large-amplitude swelling while subsequent exposure to hypertonic medium causes contraction (5,6) (Fig. 3). Swelling results in the distention and usually the disruption of the outer mitochondrial membrane (7) (Fig. 3). The inner membrane unfolds during swelling but, in most cases, remains intact. During contraction, the inner membrane refolds while the outer membrane becomes detached from the inner membrane (7). In some cases, outer membrane fragments remain attached to the inner membrane (7).

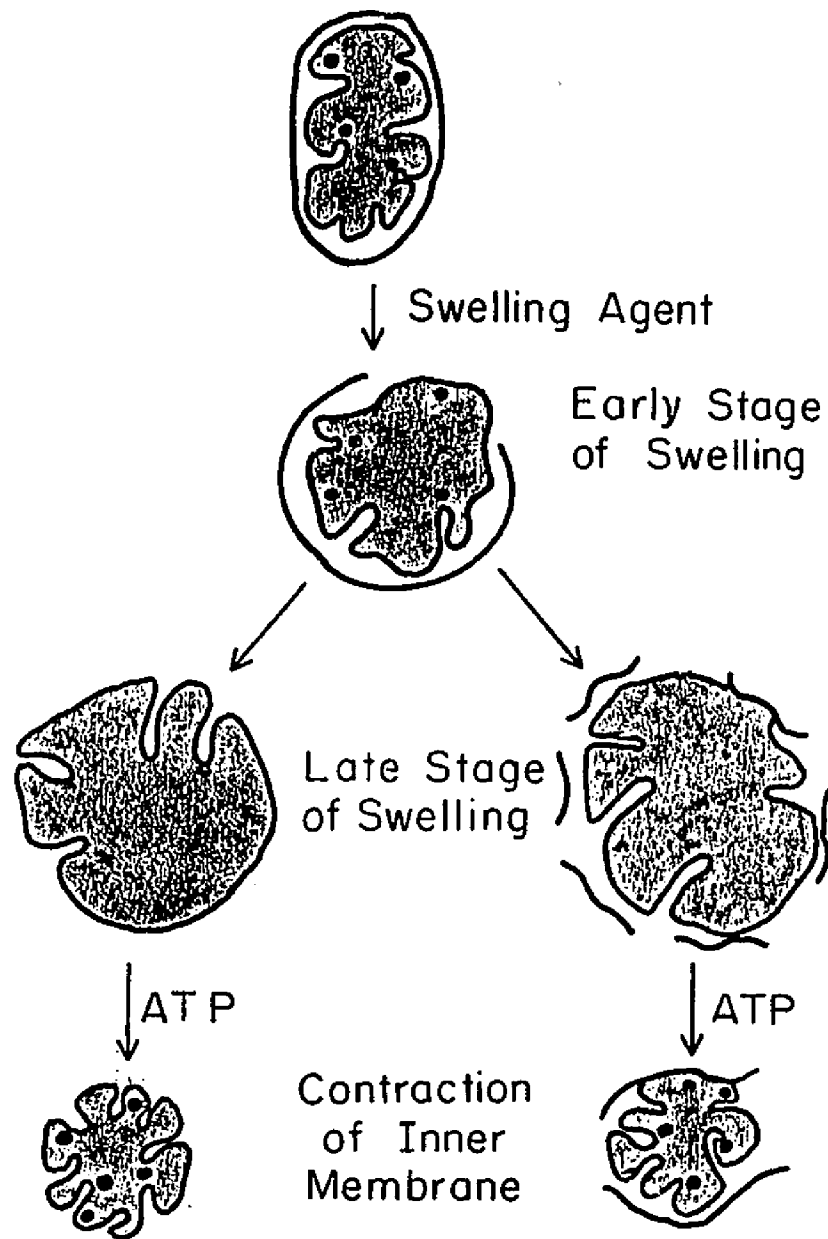


Figure 3: Conformational changes in the mitochondrion during large-amplitude swelling and contraction.

Although the changes caused by swelling to the inner membrane can be reversed, the outer mitochondrial membrane is destroyed. Thus, large-amplitude swelling in which the outer membrane is disrupted is an irreversible process.

Oxidative Phosphorylation. The most important function of mitochondria is to convert energy obtained from the oxidation of substrates into energy stored in the form of a phosphate bond in adenosine triphosphate (ATP) which may be utilized upon hydrolysis. Normal mitochondrial functioning is therefore dependent on the coupling of oxidation and phosphorylation.

The free energy of oxidation necessary to drive the process of oxidative phosphorylation is derived principally from oxidation of fatty acids (beta-oxidation) (8) and citric acid cycle intermediates (9). The energy obtained from oxidation is stored as reducing potential in the form of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂). These reducing equivalents are then used to drive mitochondrial electron transport.

The electron transport chain (ETC) is located in the inner mitochondrial membrane (10). NADH and FADH₂ are utilized at different sites on the ETC (Fig. 4). NADH, produced by oxidases distinct from the ETC, transfers electrons to the chain at NADH-oxidase (or NADH dehydrogenase), which is called complex 1 (11). FADH₂, on the other hand, is produced directly on the ETC through

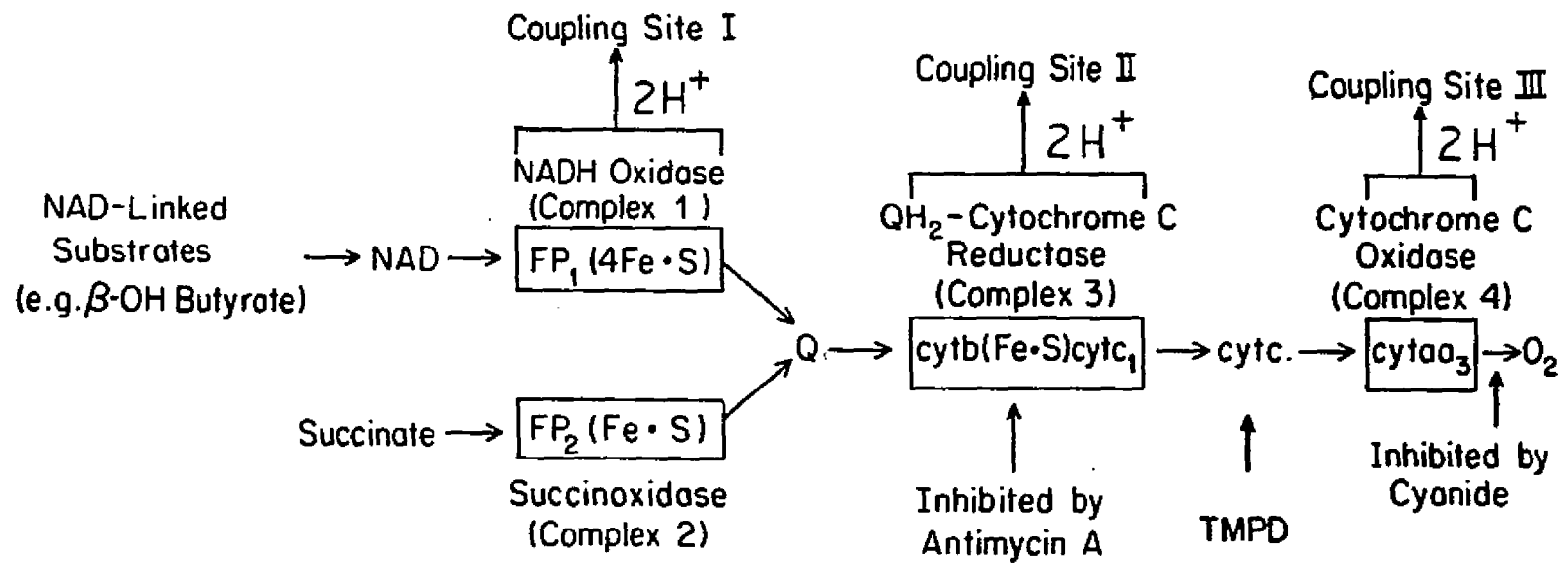


Figure 4: The mitochondrial electron transport chain.

oxidation of succinate by succinoxidase (or succinate dehydrogenase), which is called complex 2 (12). Both of these complexes transfer electrons to coenzyme Q (ubiquinone) (13) which is the first of two mobile carriers on the ETC that act as shuttles between respiratory complexes (1). According to the classical description of the ETC, electrons are then transferred in a linear fashion through coenzyme Q-cytochrome c reductase (complex 3) to cytochrome c (14,) which is the second mobile carrier on the ETC (1). Finally, electrons are transferred from cytochrome c to cytochrome oxidase (complex 4) which reduces oxygen, the terminal electron acceptor, to water (15).

A significant amount of evidence has been presented that contradicts the linear flow of electrons through complex 3 depicted in figure 4 (16-18). An alternative model, termed the protonmotive Q cycle (19) (Fig. 5), has been proposed to account for the observed discrepancies from the classical theory of linear electron transport. In this scheme, ubiquinol (QH_2 , reduced form of coenzyme Q) is oxidized to a semiquinone (Q^\cdot , radical form of coenzyme Q) via transfer of one electron to an iron-sulfur protein which then reduces cytochrome c_1 . The semiquinone is then oxidized to ubiquinone (Q, oxidized form of coenzyme Q) by transfer of one electron to cytochrome b-566, which then reduces cytochrome b-562. These reactions are believed to

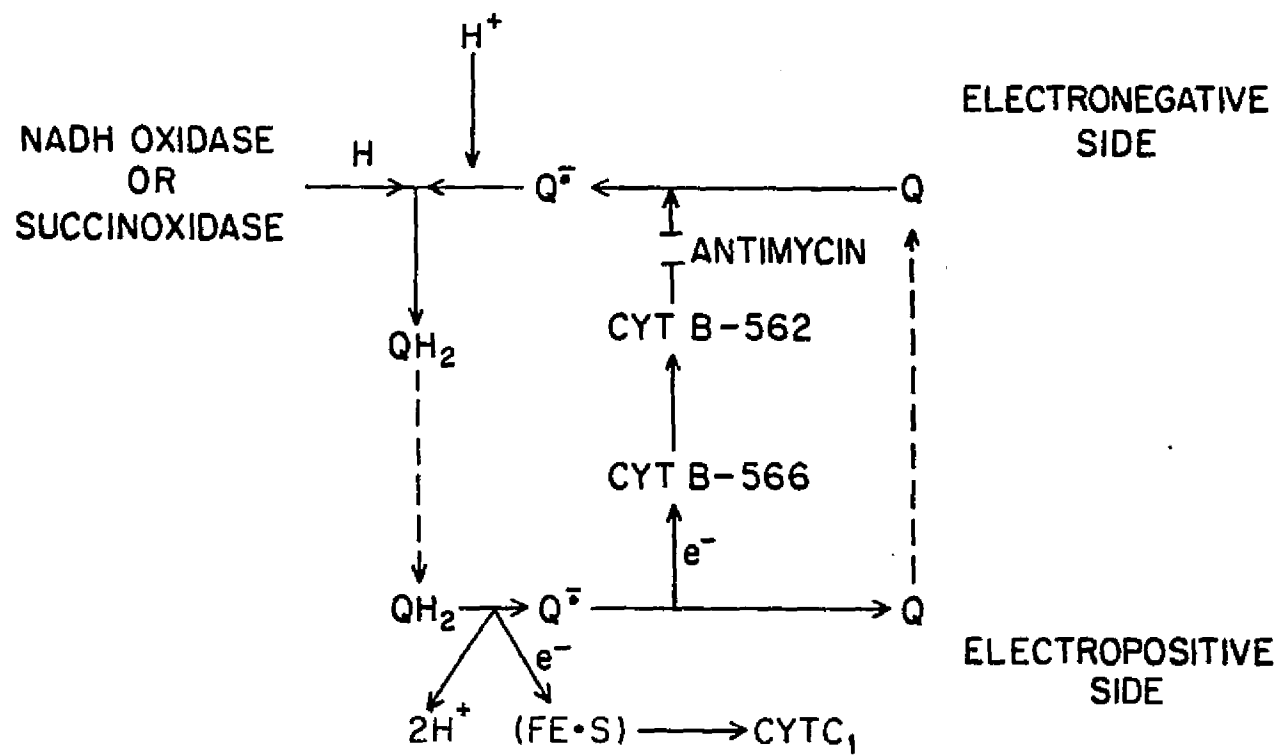


Figure 5: The protonmotive Q cycle.

occur on the electropositive side of the inner membrane. Ubiquinone, on the electronegative side of the inner membrane, is then reduced to a semiquinone by cytochrome b-562. Either NADH-oxidase or succinoxidase then reduces the semiquinone to ubiquinol to continue the cycle. Thus, according to the Q-cycle model, there are two separate binding domains for ubiquinone, located on opposite sides of the inner membrane. Another model, termed the SQ-cycle (20), suggests that there is only one binding domain for ubiquinone, and the semiquinone radical of coenzyme Q acts as both an oxidant and reductant of cytochrome b. Recent kinetic evidence has provided support for the original Q-cycle hypothesis (21). Thus, electron flow through complex 3 is not linear and is believed to occur via the scheme shown in Fig. 5. The most important aspect of the Q-cycle model is that it explains how two protons are pumped per electron transferred through coenzyme Q-cytochrome c reductase.

The chemiosmotic theory proposes that electron transport and the production of ATP from adenosine diphosphate (ADP) and inorganic phosphate are coupled through an electrochemical gradient across the inner mitochondrial membrane (22). As electrons flow down the ETC, there are three sites at which the change in redox potential is sufficiently large to obtain enough energy to pump two protons from the electronegative side of the inner

membrane (matrix) to the electropositive side (intermembrane space). These sites, termed coupling sites I, II and III, are found in complexes 1, 3 and 4 (Fig. 4). Thus, energy is expended to pump protons against an electrochemical gradient. The production of ATP is then driven by a flow of protons from the intermembrane space through H^+ -ATPase to the matrix.

The rate of respiration has been proposed to be a function of the overall thermodynamic driving force on the electron transport chain, $2\Delta E_h - n\Delta p$, where ΔE_h is the difference in redox potential across the ETC, Δp is the protonmotive force and n is the H^+/O stoichiometry of the ETC (23). Thus, a decrease in Δp will result in an increase in respiration rate. Both classical uncouplers (which imbed themselves in the inner membrane and shuttle protons from the intermembrane space to the matrix) and ADP (which also allows protons to flow across the membrane because it is a substrate for H^+ -ATPase) cause a decrease in Δp and stimulate respiration (24,25). This decrease in Δp is believed to stimulate respiration via an increase in the rate of proton pumping; this results in an increase in the rate of electron transport due to the coupling of the ETC with proton pumping (26).

Measurement of Mitochondrial Respiration. Measurement of the integrity of mitochondria is based on the coupling between respiration and phosphorylation. Mitochondrial

respiration is normally measured polarographically using a Clark-type oxygen electrode (27). Two different measurements are used to quantitate the integrity of mitochondrial preparations. The respiratory control ratio (RCR) refers to the ratio of state 3 respiration (high concentration of both substrate and ADP) to state 4 respiration (high concentration of substrate, low concentration of ADP); the rate of state 4 respiration is measured after ADP has been consumed (Fig. 6) (27). In tightly coupled mitochondria, the rate of state 4 respiration is slow; a rapid increase in oxygen uptake is observed upon the addition of ADP. If no stimulation of respiration rate is observed after adding ADP, the RCR equals 1, indicating that electron transport is completely uncoupled from phosphorylation. In Fig. 6, the RCR of the preparation is 5.8. RCR values below 3 are considered to be indicative of a poor preparation. The duration of state 3 respiration is dependent on the amount of ADP added to the mitochondrial suspension. The ADP:O ratio, another measurement of mitochondrial integrity, takes advantage of the fact that the concentration of oxygen consumed is proportional to the amount of ADP converted to ATP (27). This ratio is calculated by dividing the moles of ADP added by the moles of O (not O₂) consumed during state 3 respiration (see ΔO in Fig. 6). In Fig. 6, the ADP:O ratio of the preparation is 1.4. For every two protons

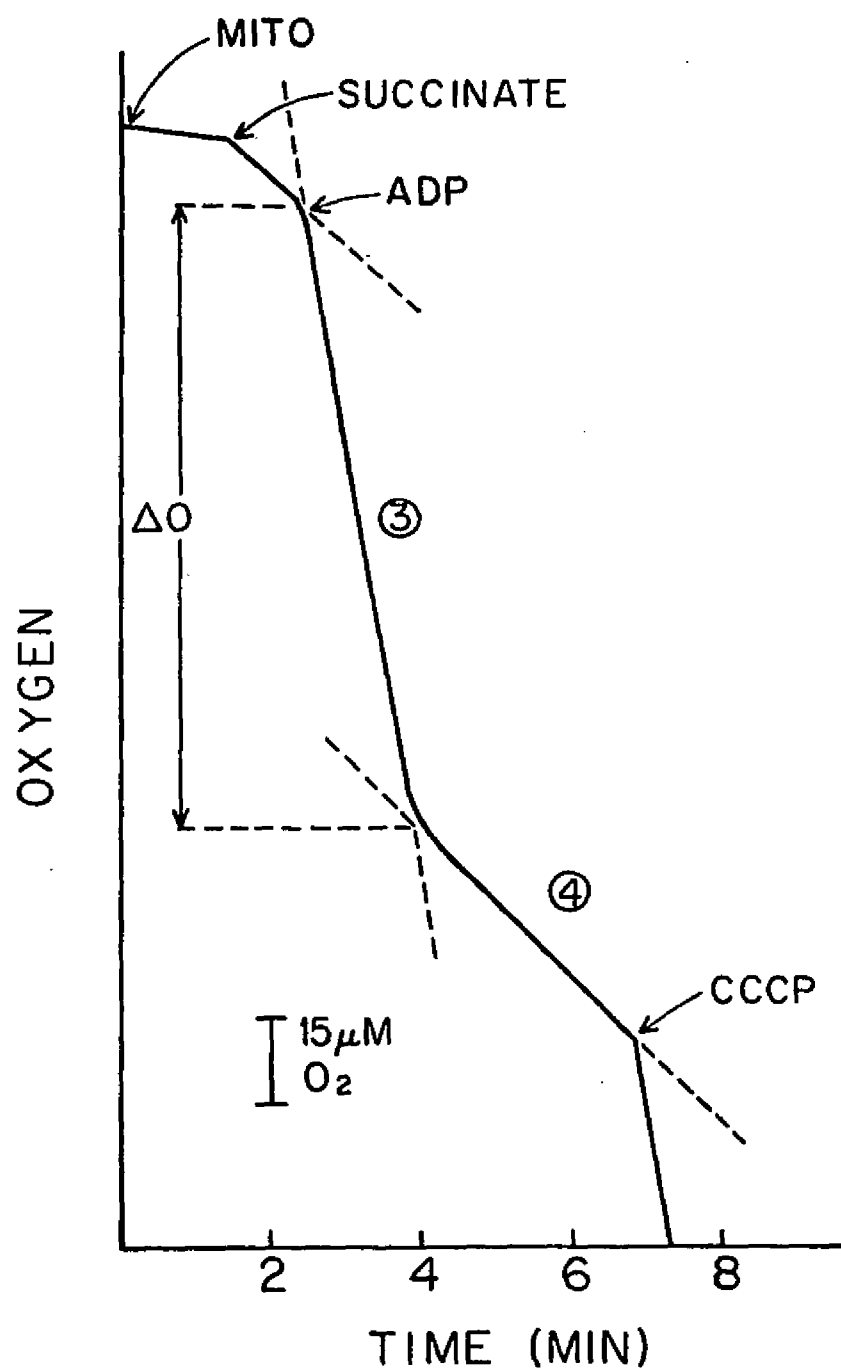


Figure 6: Measurement of respiratory control ratio (RCR) and ADP:O ratio in respiring mitochondria. RCR = rate of oxygen uptake during state 3 respiration/rate of oxygen uptake during state 4 respiration. ADP:O ratio = nmoles ADP added/nmoles O used during state 3 respiration.

pumped against the electrochemical gradient, one ADP should theoretically be converted to ATP (28). Thus, the theoretical ADP:O ratio is 3 when NAD-linked substrates are used since six protons are pumped per NADH utilized. The theoretical ADP:O ratio is 2 when succinate is the substrate since only four protons are pumped per FADH_2 consumed.

Characterization of Toxins as Inhibitors of Mitochondrial Function. Numerous toxins have been shown to directly disrupt mitochondrial oxidative phosphorylation by inhibiting either electron transport, ATP synthesis or substrate/ion transport or by uncoupling respiration from phosphorylation (29). Mitochondria are essential for the normal functioning of cells and destruction of this organelle results in cell death (30). Mitochondria can be implicated as a major target of toxicity by demonstrating that a toxin is a mitochondrial inhibitor.

A toxin can be characterized as a mitochondrial inhibitor by examining its effect on mitochondrial respiration. The most commonly used technique for accomplishing this is the measurement of oxygen uptake. If a stimulation in oxygen uptake is observed when a toxin is added to mitochondria during state 4 respiration and the degree of stimulation is independent of added ADP, then the toxin may be classified as an uncoupler of respiration (31). If a decrease in oxygen uptake is observed when a

toxin is added during state 3 respiration, then the toxin may be an inhibitor of either the respiratory chain (32), ATP synthesis (33), substrate/ion transport (34,35) or a combination of these. A classical uncoupler such as carbonyl cyanide m-chlorophenylhydrazone (CCCP) may be added to determine if respiration can be stimulated in the presence of the toxin. If respiration is not stimulated, then the toxin is a respiratory chain inhibitor. If inhibition of respiration is completely reversed by CCCP, then the toxin is an inhibitor of ATP synthesis. It is possible to distinguish between an inhibitor of ATP synthesis and an inhibitor of substrate/ion transport by measuring ATPase activity in submitochondrial particles in the presence of the toxin. ATPase activity is affected by inhibitors of ATP synthesis whereas it is unaffected by inhibitors of substrate/ion transport. These latter inhibitors are also characterized by the fact that they do not completely inhibit oxygen consumption and ATP synthesis in whole mitochondria (36).

If a toxin is characterized as a respiratory chain inhibitor, the site of interaction on the ETC may be probed by adding the toxin during both NAD- and FAD-linked respiration. If NAD-linked respiration is inhibited but FAD-linked respiration is not, then the site of interaction is complex 1. If the converse is true, then the site of interaction is complex 2. Inhibition of both types of

respiration suggests that the toxin interacts with a site downstream on the respiratory chain. N,N,N',N' -tetramethyl-p-phenylenediamine (TMPD) is an artificial electron donor that transfers electrons to cytochrome c (Fig. 4) (37). If release of inhibition of respiration is observed in the presence of TMPD, then the toxin interacts at complex 3. If no stimulation of oxygen uptake is observed with TMPD, then the toxin inhibits complex 4.

Difference spectroscopy is a technique that employs measurement of the spectral properties of chain components in the visible region (400 - 700 nm) to determine the site of interaction of a toxin with the respiratory chain (38). The absorbance of respiring mitochondria (or respiring submitochondrial particles) treated with the toxin is compared to untreated, oxidized mitochondria (or submitochondrial particles). If a toxin interacts specifically with either complex 1 or complex 2, no accumulation of reduced cytochromes will be observed. If a toxin interacts with complex 3 in a manner analogous to antimycin A, an inhibitor of proton pumping site II, then an accumulation of only cytochromes b will be observed (Fig. 7A). If a toxin interacts with complex 4, then cytochromes b, c and a will all accumulate in their reduced form (Fig. 7B). Thus, difference spectroscopy is a useful probe of toxin-ETC interactions.

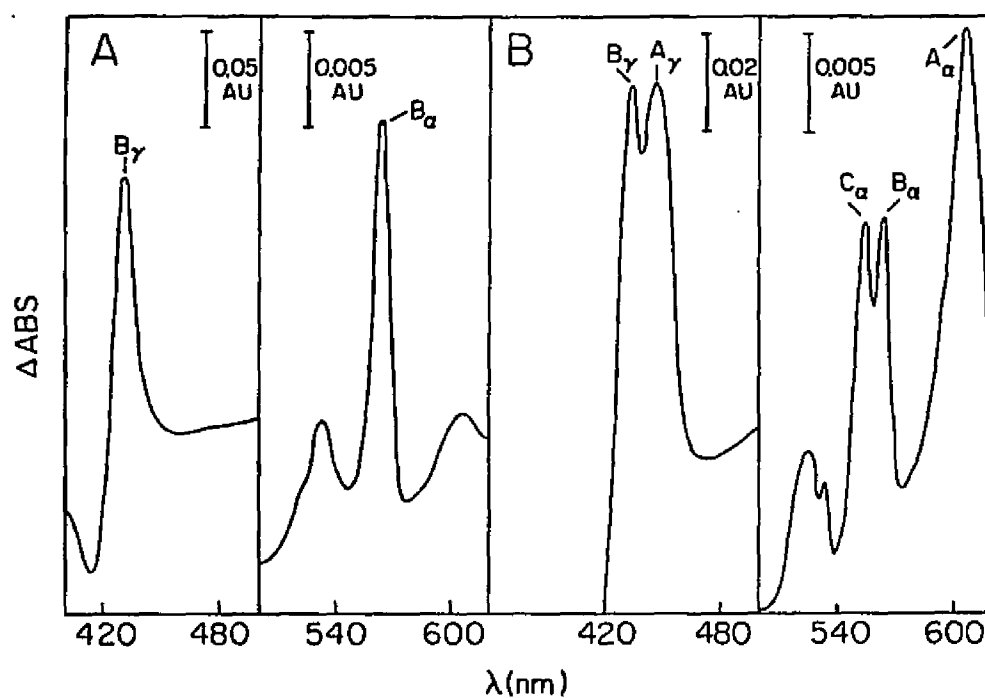
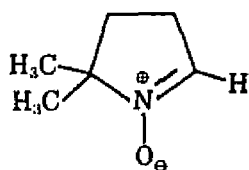
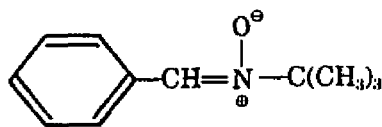


Figure 7: Accumulation of reduced cytochromes in respiring SMP blocked at complex 3 (A) and complex 4 (B). (A) Accumulation of cytochrome b resulting from the treatment of respiring SMP with antimycin A. (B) Accumulation of cytochromes a, b and c resulting from the treatment of respiring SMP with sodium cyanide. Greek symbols refer to the individual absorption bands of the cytochromes.

Electron spin resonance spectroscopy (ESR), a technique used to detect paramagnetic species, can also be used to probe the site of interaction of toxins with the respiratory chain because a buildup of reduced coenzyme Q results in a one-electron reduction of O_2 to $O_2^{\cdot -}$ (superoxide radical) via the semiquinone radical of coenzyme Q (39). Direct detection of radicals by ESR depends on the buildup of a steady-state concentration of these species. Due to their reactivity, many radicals have a short lifetime. Thus, it is sometimes difficult to obtain a sufficient concentration of radicals in biological systems to allow for direct detection by ESR. This problem has been solved by the introduction of spin traps (40-45). The most common spin traps are nitrones. Two of the most popular nitrones for ESR spin-trapping experiments are 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and alpha-phenyl N-tert-butyl nitron (PBN).

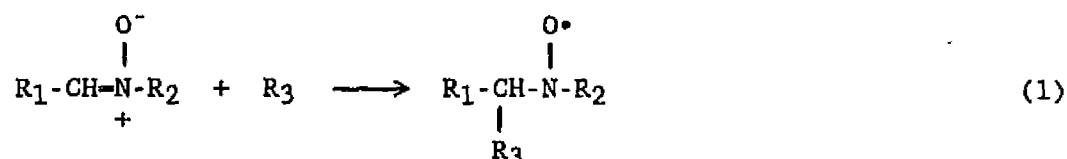


DMPO

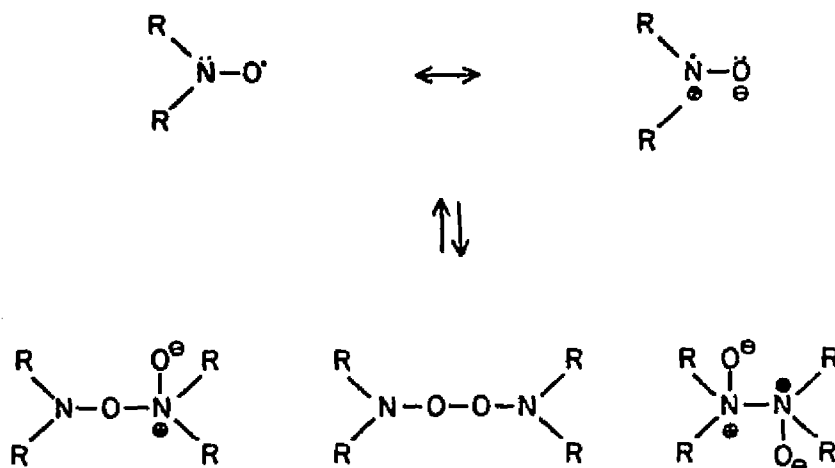


PBN

Nitrones react with short-lived free radicals to produce nitroxide (or aminoxyl) radicals which are termed "stable" radicals (46) (Equation 1).



The stability of nitroxides is due to resonance stabilization of the unpaired electron in the monomer and the fact that strong bonds cannot be formed in the dimer (46).



The actual stability of the nitroxide is dependent on the free radical that is trapped.

In an electromagnetic field, there is a certain frequency (termed the resonance frequency) at which a free electron will absorb an amount of energy equal to the energy-level separation between its two possible spin

states (47). In an ESR spectrum, which is normally recorded as the first derivative spectrum, this absorption results in a single line (Fig. 8A). The interaction of an unpaired electron with a nuclear species possessing nuclear spin (e.g., $I = 1/2, 1, 3/2$, etc.) results in a splitting of the single line into multiple lines of equal intensity. The number of lines observed from the interaction of an unpaired electron with a single nucleus is equal to $2I + 1$. For example, hydrogen ($I = 1/2$) splits the single line into two lines (Fig. 8B) while interaction with nitrogen ($I = 1$) results in three lines (Fig. 8C). The quantity hA_0 , where h is Plank's constant and A_0 is the isotropic hyperfine coupling constant, is a measure of the interaction energy between the electron and the nucleus. In an ESR spectrum, the separation between lines due to interaction of an unpaired electron with a single nucleus is defined as the hyperfine splitting constant (a). The hyperfine splitting and coupling constants are related by the equation $a = hA_0/g\beta$, where h is Plank's constant, g is the g factor, and β is the Bohr magneton (47). These constants are useful for the identification of radicals since most ESR spectra arise via coupling of electron and nuclear spin.

In the case of both DMPO- and PBN-radical adducts, splittings are observed due to the interaction of the unpaired electron with the both the nitrogen and beta-hydrogen on the trap. If these splittings are

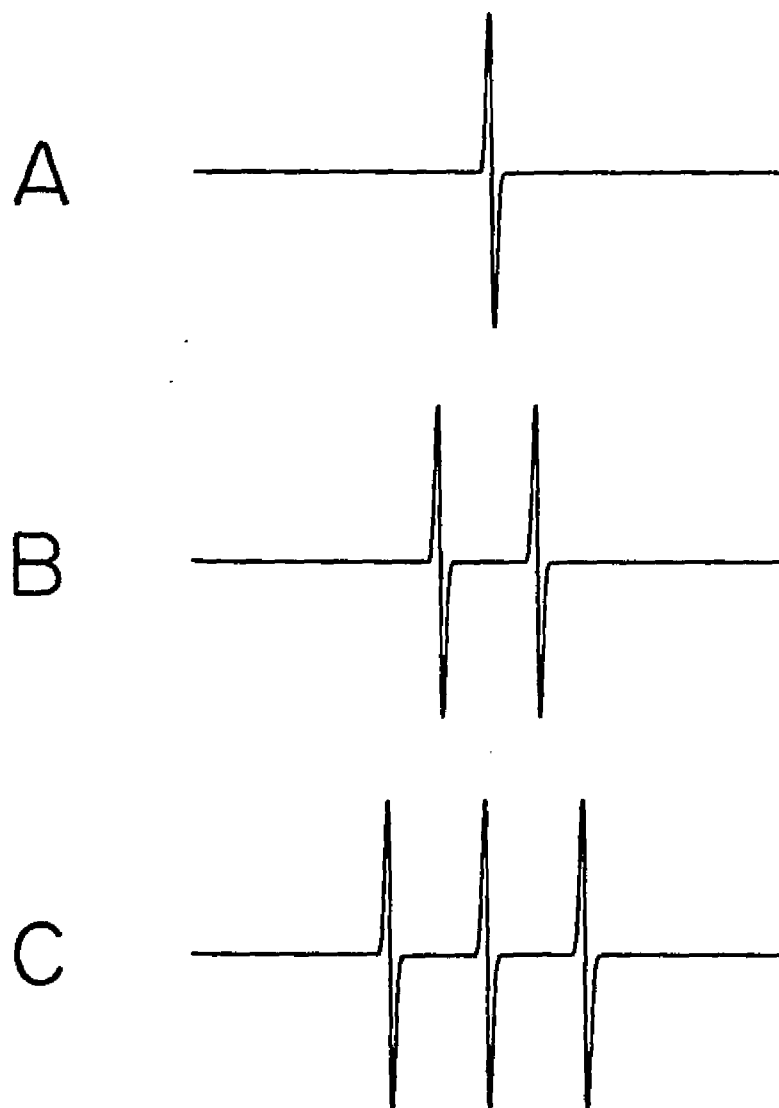


Figure 8: Effect of nuclear spin on the splitting of the ESR signal of an unpaired electron. (A) ESR signal of an unpaired electron. (B) Effect of a single hydrogen nucleus on A where $a_H = 1.5$ mT. (C) Effect of a single nitrogen nucleus on A where $a_N = 1.5$ mT. Sweep width = 9.0 mT.

significantly different, the result is a six-line ESR signal (Fig. 9). The interaction energy between the unpaired electron and beta-hydrogen nucleus is greater in a DMPO adduct (Fig. 9A) than it is in a PBN adduct (Fig. 9B), resulting in larger hydrogen splittings. Thus, the ESR spectrum of a DMPO adduct provides more information about the trapped species than does a PBN adduct.

The ESR spin-trapping technique can be used in a respiring mitochondrial system to determine whether a chain inhibitor blocks electron flow on the oxidase side of coenzyme Q. For example, when respiring beef heart mitochondria are treated with antimycin A, an inhibitor of complex III (Fig. 4), in the presence of DMPO, the formation of a superoxide-DMPO adduct is observed (48). This technique can also provide direct evidence for the interaction of a toxin with mitochondria via a radical pathway. For example, tert-butyl hydroperoxide (TBHP) has been shown to damage mitochondria by a non-radical pathway involving calcium cycling (49-51). We have used the ESR spin-trapping technique to show that TBHP also interacts with mitochondria by a radical pathway (52). Thus, ESR shows great promise for the study of toxin/mitochondrial interactions.

In addition to disturbing mitochondrial oxidative phosphorylation, certain toxins also induce changes in the morphology of mitochondria (29). These changes can be

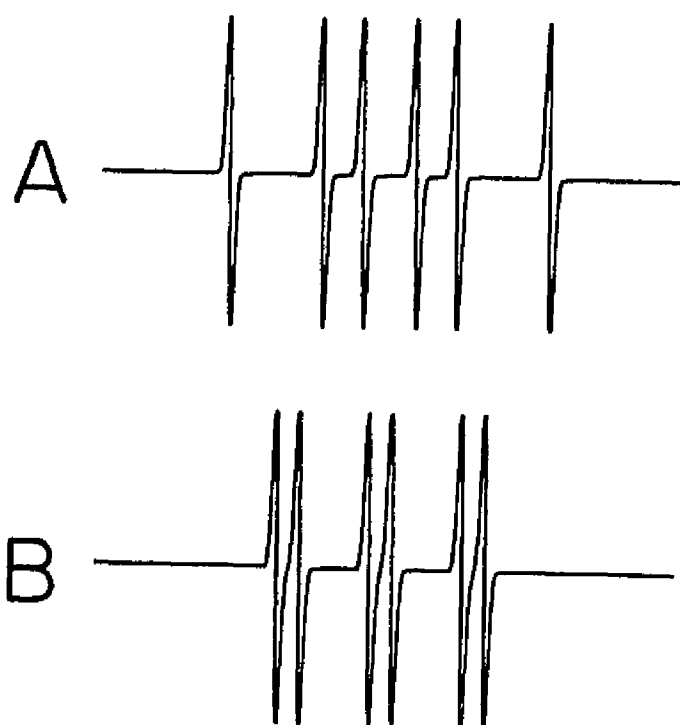


Figure 9: Gaussian Simulations of ESR spectra of methyl radical adducts of DMPO and PBN. (A) Methyl-DMPO adduct signal generated using hfsc of $a_N = 1.43$ mT and $a_H = 2.05$. (B) Methyl-PBN adduct signal generated using hfsc of $a_N = 1.42$ mT and $a_H = 0.34$ mT. Sweep width = 9.0 mT.

measured by following the decrease in turbidity of a mitochondrial suspension using a visible spectrophotometer. This physical phenomenon is referred to as mitochondrial swelling. As stated previously, a change in the energy state of the mitochondrion from high to low energy results in a conversion from a condensed to an orthodox conformation (3). This conformation change is the basis of reversible low-amplitude, energy-linked mitochondrial swelling (4). A change from condensed to orthodox conformation also occurs during large-amplitude swelling, but this type of swelling is not necessarily related to the metabolic state of the mitochondrion. Lysis of the outer mitochondrial membrane also occurs in most cases of large-amplitude swelling making this process irreversible (7). Toxin-induced mitochondrial swelling has been shown to occur by a number of different mechanisms. For example, calcium cycling (49-51), lipid peroxidation (53), and oxidation of sulfhydryl groups (54) have all been implicated in this process. Mitochondrial swelling is an important indicator of the toxicity of a compound since this change of morphology and loss of mitochondrial membrane potential have been shown to precede cell death (30).

By using the techniques described above, it is possible to determine if a compound is a mitochondrial toxin and, if so, to probe the mechanism of toxicity.

PHYSIOLOGICAL SIGNIFICANCE OF BENZOYL PEROXIDE

Preparation and Uses of Benzoyl Peroxide. Benzoyl peroxide (BPO) is a free radical-generating compound that is commonly used in the chemical industry as an initiator of radical processes and in the pharmaceutical industry as an active ingredient in many acne medicines. BPO is prepared by treatment of benzoyl chloride with either hydrogen peroxide in alkaline medium (55) or sodium peroxide in acetone (56).

Effect of BPO on Skin. Since BPO is used in acne medicines, its effect on skin is of interest. When used undiluted or at high concentrations, BPO is a potent skin irritant (57) and causes epidermal hyperplasia (58). These findings prompted further study into the action of BPO on skin with emphasis on its ability to participate in carcinogenesis.

Three Stage Model of Skin Carcinogenesis. Skin carcinogenesis can be divided into initiation, promotion and progression stages. Tumor initiation is an irreversible step that arises from the interaction of a carcinogen with DNA resulting in gene alteration (59). During the promotion stage, the altered genetic material of the initiated cell becomes expressed through selection and clonal expansion (60). This process is facilitated by the ability of the tumor promoter to both increase the rate of terminal differentiation of the non-initiated cells and

exhibit toxic effects on these cells (61). The result of initiation followed by promotion is commonly the formation of a high yield of benign tumors. The progression of tumors from a benign to malignant neoplasm occurs in the final stage of carcinogenesis; this transformation is proposed to involve further genetic changes caused by mutagenic agents (62,63). This model of carcinogenesis has been shown to exist in a number of systems including the liver (64).

Activity of Peroxidic Compounds in Tumor Promotion.

Although BPO is inactive as a tumor initiator or complete carcinogen (65), it is both an effective skin tumor promoter (66) and enhancer of progression (67). Several other peroxidic compounds are also active as tumor promoters; cumene hydroperoxide (CHP), decanoyl peroxide, dicumyl peroxide, and lauroyl peroxide are strong promoters while tert-butyl hydroperoxide (TBHP), hydrogen peroxide, and methyl ethyl ketone peroxide are less active (58,68-70). It has been suggested (58) that detoxification of H_2O_2 and organic hydroperoxides by cellular catalase and glutathione peroxidase (71) may prevent some of the intracellular effects of these compounds resulting in a lowered tumor promoting activity. Organic peroxides, unlike hydroperoxides, are not metabolized by glutathione peroxidase (72); therefore, the effective cellular concentration of the peroxides may be higher than the

hydroperoxides, resulting in a higher tumor promoting activity. Although this theory is reasonable, it does not explain the fact that cumene hydroperoxide is a good tumor promoter.

Induction of Morphological and Biochemical Changes in Skin by BPO. Tumor promoters have been found to cause a number of morphological and biochemical changes in skin (73). Of these effects, the induction of epidermal cell proliferation, dark basal keratinocyte production, and ornithine decarboxylase activity show the best correlation with tumor promoting activity (74-79).

Keratinocytes are electron-dense, ribosome-rich basal cells which are observed during inflammation and epidermal hyperplasia (77-79). BPO is an effective hyperplasiogenic agent when applied to rodent skin and results in a sustained production of keratinocytes (58). BPO inhibits metabolic co-operation in cultured human keratinocytes (80) and has been shown to produce single strand breaks in cultured human bronchial epithelial cells (81), mouse keratinocytes (82) and keratinocyte-derived cell lines (83). The latter effect may be important in the role of BPO as an enhancer of tumor progression (67) since this stage of carcinogenesis is believed to involve genetic changes (62,63).

A recent study has shown that mouse keratinocytes derived from initiated skin or papillomas are resistant to

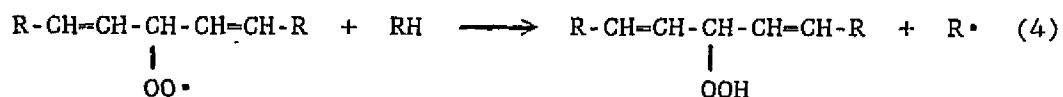
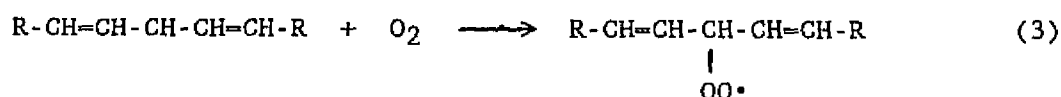
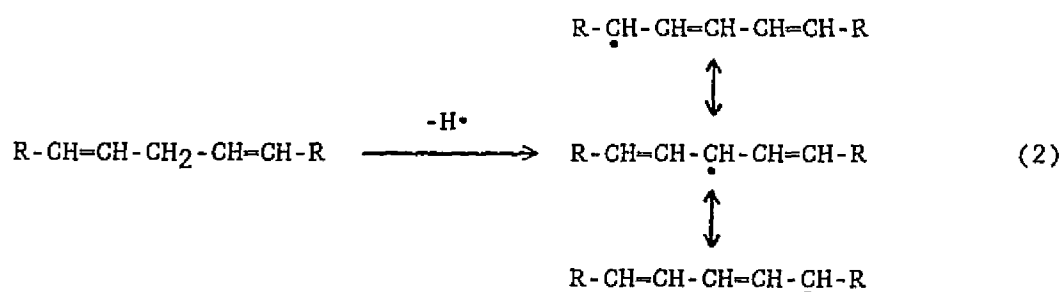
DNA strand breakage by BPO and suggests that this may be important with regard to BPO-mediated tumor promotion (84). As stated previously, expression of initiated cells is facilitated by the ability of the tumor promoter to both increase the rate of terminal differentiation of the non-initiated cells and exhibit toxic effects on these cells (61). The induction of single strand breaks in non-initiated keratinocytes by 12-O-tetradecanoyl phorbol-13-acetate (TPA), a strong tumor promoter, has been related to its ability to induce terminal differentiation (82). Induction of terminal differentiation is proposed to occur via activation of protein kinase C (85). Although BPO has been shown to activate protein kinase C in epidermal cell membranes (86) and induce single strand breaks in normal basal keratinocytes (non-initiated), it does not induce terminal differentiation in these cells (82). Thus, resistance to differentiation may provide initiated cells with a selective advantage in phorbol ester-mediated tumor promotion while resistance to cytotoxicity may provide this advantage in BPO-mediated tumor promotion (82).

Involvement of Free Radicals in Tumor Promotion and Peroxide-Induced Cytotoxicity. Free radicals have been implicated in the tumor promotion stage of carcinogenesis (87,88). As stated previously, one of the roles of a tumor promoter is to exhibit toxic effects on uninitiated cells (61). The ability of BPO to induce lipid peroxidation in

human epidermal keratinocytes has been probed to determine whether this radical process is related to cell damage caused by BPO (89). BPO does not induce lipid peroxidation in these cells (89); therefore, the cytotoxicity of BPO must be attributed to a process distinct from lipid peroxidation. Possibilities include oxidation of proteins (90), production of strand breaks in DNA (81-83), or the inhibition of mitochondrial respiration (91).

PHYSIOLOGICAL SIGNIFICANCE OF HYDROPEROXIDES

Formation of Hydroperoxides. Although cyclic peroxides are still believed to be formed in the peroxidation of polyunsaturated fatty acids (PUFA) (92,93), the primary reaction products of autoxidation are hydroperoxides. The mechanism for the autoxidation of PUFA commonly accepted today was first proposed by Bolland and Koch in 1945 (94) (Equations 2-4).

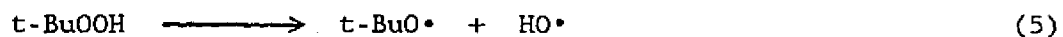


Equation 2 represents the initiation step in which a reactive species abstracts a hydrogen atom from an alpha-methylene group of a PUFA. In biologically relevant PUFA, these methylene groups are doubly allylic due to the

position of the double bonds (e.g. linoleic acid is a 9,12 unsaturated fatty acid); the doubly allylic hydrogens in these compounds are more labile than the alpha-methylene hydrogens found in mono-unsaturated compounds (e.g. oleic acid) and are therefore more susceptible to abstraction. For this reason, PUFA are more easily oxidized than are either saturated or mono-unsaturated fatty acids. The radical formed by abstraction of a hydrogen atom (Equation 2) can isomerize to form three resonance hybrid free radicals. Each of these carbon-centered radicals can then react readily with oxygen to form a peroxy radical (Equation 3). The peroxy radical then abstracts a hydrogen atom from another PUFA molecule to form a hydroperoxide and a new carbon-centered radical that is capable of continuing this chain reaction (Equation 4). Equations 3 and 4 are the propagation steps in autoxidation. Termination of the chain results when either two radicals react to form non-radical products or when a radical abstracts a hydrogen atom from a compound that yields a less-reactive radical.

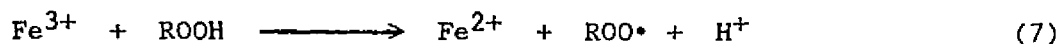
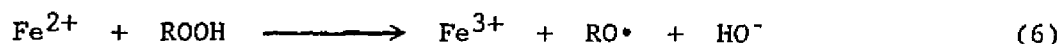
The occurrence of autoxidation (or lipid peroxidation) in a biological system was first observed by incubating homogenates of liver, kidney, and brain under aerobic conditions and estimating the extent of peroxidation by the thiobarbituric acid (TBA) test (95,96).

Decomposition of Hydroperoxides. The bond dissociation energy (BDE) of the oxygen-oxygen bond in simple alkyl hydroperoxides is fairly strong. For example, the BDE of tert-butyl hydroperoxide (TBHP) is 43 kcal/mole (97). Thus, the rate constant for the uncatalyzed, unimolecular decomposition of TBHP (Equation 5) at 37°C is



$5 \times 10^{-16} \text{ sec}^{-1}$, and the half life for TBHP at 37°C is about 10^9 years (98). Since the BDE of the oxygen-oxygen bond should be similar for all simple alkyl hydroperoxides (99,100), uncatalyzed thermal decomposition is not likely to be an important route for breakdown of these compounds under physiological conditions (101).

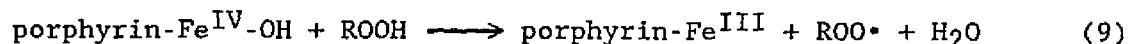
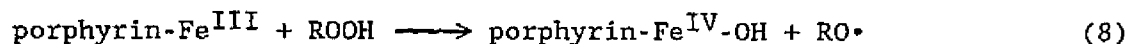
Transition metal complexes readily catalyze the decomposition of hydroperoxides in vitro via Fenton reactions (102-104) to yield alkoxyl and peroxy radicals (Equations 6 and 7).



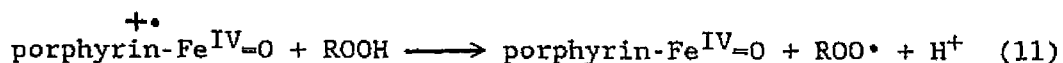
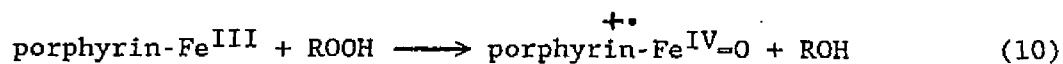
Decomposition of hydroperoxides via metal-catalyzed redox reactions is expected to be an important process in biological systems since hydroperoxides are degraded by metals from endogenous sources such as heme proteins (105-107). Iron released from ferritin and hemosiderin

initiates lipid peroxidation (108-110), so these iron sources may also be involved in radical generation by hydroperoxides in vivo.

Three different mechanisms have been proposed to account for the radicals observed when heme proteins are treated with hydroperoxides. Heme proteins are believed to catalyze the decomposition of hydroperoxides via Fenton reactions (Equations 6 and 7) (111,112). An alternative mechanism has been proposed in which the initial event is homolytic cleavage of the oxygen-oxygen bond of the hydroperoxide molecule by the heme (113) (Equations 8 and 9); this reaction results in the formation of an alkoxyl radical and heme-bound hydroxyl radical.



The products of both of these mechanisms is an alkoxyl radical, a peroxy radical and water. Several heme proteins are also believed to catalyze the decomposition of hydroperoxides via a peroxidase-type mechanism resulting in the formation of peroxy radicals (114-117) (Equations 10 and 11).



Radicals produced via the decomposition of lipid hydroperoxides by endogenous iron may cause cellular damage through interaction with DNA (118,119), proteins (120,121) or lipids (to yield further lipid peroxidation) (122,123).

Involvement of Hydroperoxides in Carcinogenesis.

Organic hydroperoxides have been shown to have varying degrees of tumor promoting activity in the initiation-promotion model of mouse epidermis; cumene hydroperoxide (CHP) is a strong promoter, TBHP and methyl ethyl ketone peroxide are weak promoters, and hydrogen peroxide is a very weak promoter (58,68-70).

The ability of hydroperoxides to induce radical production in mouse keratinocytes, a target cell for tumor promoters, has been measured by the ESR spin-trapping method; both CHP and TBHP are effective radical generators in these cells (124). Surprisingly, TBHP does not induce lipid peroxidation in keratinocytes (89). Therefore, radicals generated by TBHP must be either readily scavenged in these cells or participate in another type of cellular damage such as modification of proteins or DNA. Lipid peroxidation is not a requisite for TBHP-induced cell death in hepatocytes (125) indicating that other types of damage must be involved in the toxicity of this compound. Kensler and Taffe have suggested that methyl radicals produced in hydroperoxide-treated keratinocytes may modulate the cell phenotype during promotion or progression by alkylating

cellular macromolecules (87). This is a reasonable hypothesis since methylation reactions are believed to be involved in eukaryotic gene expression (126).

The fact that TBHP, unlike BPO, does not reduce the cell viability of normal mouse keratinocytes (89) may provide a rationale for the difference in skin tumor-promoting activity of these two peroxidic compounds. Since tumor cells are resistant to lipid peroxidation (127,128) and neither BPO nor TBHP initiate this radical process in normal cells (89), it can be proposed that lipid peroxidation is not an important process in peroxide-mediated tumor promotion in skin.

Possible Involvement of Mitochondria in Carcinogenesis and Mechanisms of Interaction of Hydroperoxides With These Organelles. Many carcinogens are known to interact preferentially with mitochondrial DNA, suggesting that the mitochondrion may be a target organelle for these compounds (129). Chemical carcinogens might transform cells by interacting at the locus of the mitochondrial membrane resulting in loss of genetic material through the membrane into the cell where it acts as an exogenous oncogenic virus (130).

Mitochondrial function is reported to be impaired in cancer cells (131); these impairments include changes in ultrastructure, defective energy-linked functions, and altered Ca^{2+} transport. Organic peroxides have been shown

to cause similar deviations in mitochondrial function. Peroxide-induced damage occurs by both radical and non-radical pathways; for example, TBHP has been shown to initiate mitochondrial swelling via a non-radical process that is dependent on Ca^{2+} cycling (49-51), but TBHP also forms free radicals in mitochondria as shown by ESR spin trapping (52). Cumene hydroperoxide can also be presumed to yield radicals in mitochondria since it is known to initiate mitochondrial lipid peroxidation (132).

TBHP and CHP have been shown to induce radical production in keratinocytes via interaction with a cytosolic species (124); no radical generating activity is observed with the mitochondrial fraction from these cells (124). Thus, peroxide-mitochondrial interactions may not be important in skin tumor promotion if the tumor-promoting ability of peroxides is, in fact, directly related to their ability to induce radical production. The three stage model of carcinogenesis (initiation, promotion and progression) has been shown to exist in a number of systems including the liver (64). In liver, peroxide-mitochondrial interactions may be important in peroxide mediated tumor-promotion since hydroperoxides induce radical production in this organelle.

Since the mechanism of action of peroxide tumor promoters is controversial, it is not known whether

mitochondrial damage is important in the peroxide-mediated promotion stage of carcinogenesis.

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CHAPTER TWO

Benzoyl Peroxide Interaction With Mitochondria: Inhibition of Respiration and Induction of Rapid, Large-Amplitude Swelling¹

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Key Words: Carcinogenesis, Electron Transfer, Free
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October 18, 1988

Dr. Lester Packer
Editor, Archives of Biochemistry and Biophysics
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1250 Sixth Avenue
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Dear Les,

Enclosed are four copies of a manuscript entitled "Benzoyl Peroxide Interaction With Mitochondria: Inhibition of Respiration and Induction of Rapid, Large-Amplitude Swelling" that we would like for you to consider for publication in Archives of Biochemistry and Biophysics.

This manuscript reports that the treatment of rat liver mitochondria with benzoyl peroxide results in inhibition of both electron and energy transfer. We present evidence to show that benzoyl peroxide inhibits electron transfer at coupling site II and that the action of this compound is intermediate between a pure chain inhibitor and ATPase inhibitor. This paper also reports that benzoyl peroxide induces rapid, large-amplitude swelling of mitochondria by a mechanism that appears to be dissociated from radical production and lipid peroxidation.

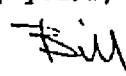
A novel aspect of this paper concerns the action of benzoyl peroxide which alters mitochondria in a manner quite different from that of other peroxide tumor promoters.

We recognize that this manuscript is lengthy. The mitochondrion has so many potential loci for interaction with peroxidic compounds that rigorous experimental proofs were required to establish our conclusions.

We hope you will favorably consider this manuscript for Archives of Biochemistry and Biophysics.

With warm regards,

Very truly yours,


William A. Pryor

Enclosure: Manuscript # 8804

ABSTRACT

When micromolar concentrations of benzoyl peroxide (BPO) are added to rat liver mitochondria, inhibition of mitochondrial NADH-oxidase and succinoxidase is observed. The addition of 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, results in only partial release of this inhibition, suggesting that BPO inhibits both electron- and energy-transfer in mitochondria. Release of inhibition is also observed when an electron donor, N,N,N',N'-tetramethyl-p-phenylenediamine, is added, suggesting that inhibition occurs on the substrate side of ferricytochrome c. When BPO is added to respiring submitochondrial particles (SMP), only reduced cytochrome b is observed to accumulate in the difference spectrum (reduced minus oxidized) in a manner analogous to that observed in the presence of antimycin A. These results indicate that BPO interacts at coupling site II between cytochromes b and c₁. When respiring SMP are treated with BPO in the presence of the spin trap 5,5-dimethyl-1-pyrroline-N-oxide, electron spin resonance signals attributable to the hydroxyl and superoxide adducts are observed. Catalase and superoxide dismutase inhibit the formation of these adducts, suggesting the involvement of both hydrogen peroxide and superoxide radicals in this process. BPO also induces rapid, large-amplitude swelling of mitochondria; the swelling is dependent on the presence

of monovalent cations but is independent of the presence of calcium, oxygen and respiratory substrate. BPO-induced swelling appears to be disassociated from radical production and lipid peroxidation.

ABBREVIATIONS

ADP, adenosine-5'-diphosphate; a-T, alpha-tocopherol; BHB, beta-hydroxy butyrate; BPO, benzoyl peroxide; BSA, bovine serum albumin; CCCP, carbonyl cyanide m-chlorophenylhydrazine; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DMPO-OH, hydroxyl adduct of DMPO; DMPO-OOH, superoxide adduct of DMPO; DMVN, 2,2'-azo-bis(2,4-dimethylvaleronitrile); DNP, 2,4-dinitrophenol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, [ethylenebis-(oxyethylenenitrilo)] tetraacetic acid; ESR, electron spin resonance; GSH, reduced glutathione; GSSG, oxidized glutathione; NEM, N-ethylmaleimide; PBN, N-tert-butyl-alpha-phenylnitrene; PMB, p-hydroxymercuribenzoate; SOD, superoxide dismutase; SMP, submitochondrial particles; TBA, 2-thiobarbituric acid; TBHP, tert-butyl hydroperoxide; TCA, trichloroacetic acid; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; Tris, 2-amino-2(hydroxymethyl)-1,3-propanediol.

INTRODUCTION

Several peroxidic compounds are active as tumor promoters; benzoyl peroxide, decanoyl peroxide, dicumyl peroxide, and lauroyl peroxide are strong promoters while tert-butyl hydroperoxide (TBHP), hydrogen peroxide, and methyl ethyl ketone peroxide are less active (1). Benzoyl peroxide (BPO) is a free radical-generating compound that is commonly used in the chemical industry as an initiator of radical processes and in the pharmaceutical industry as an active ingredient in many acne medicines. In addition to being a skin-tumor promoter (2), BPO is also an enhancer of tumor progression (3). At concentrations equal to or greater than 0.4 M, BPO is a potent skin irritant (4) and causes epidermal hyperplasia (5). During BPO-induced hyperplasia, a sustained production of dark basal keratinocytes is observed (5). The number of induced keratinocytes has been shown to correlate well with the tumor-promoting activity of several promoters (6,7).

Many promoters, such as the phorbol esters, are almost exclusively membrane-active agents. The cell membranes of many tissues contain high affinity, specific receptor proteins that interact with tumor promoters (8,9). The mitochondrion is a complex organelle consisting of membrane-bound multienzyme systems. In cancer cells, mitochondrial function is reported to be impaired (10); these impairments include changes in ultrastructure,

defective energy-linked functions, and altered Ca^{2+} transport. Prooxidant promoters, including organic peroxides, have been shown to cause similar deviations in mitochondrial function. Although peroxidic tumor promoters are known to impair normal mitochondrial function, it remains to be established whether such impairment is involved in the tumor promotion process.

Free radicals have been implicated in tumor promotion (11,12). Peroxide-induced mitochondrial damage occurs by both radical and non-radical pathways; for example, TBHP has been shown to initiate mitochondrial swelling via a non-radical process that is dependent on Ca^{2+} cycling (13-15), but TBHP also forms free radicals in mitochondria as shown by ESR spin trapping (16). Cumene hydroperoxide also can be presumed to yield radicals in mitochondria since it is known to initiate mitochondrial lipid peroxidation (17). TBHP and cumene hydroperoxide have also been shown to induce radical production in keratinocytes (18). The radical process of lipid peroxidation has been shown to be inducible in keratinocytes but is unrelated to cell damage (19); BPO is cytotoxic to these cells but does not induce lipid peroxidation (19). Thus, it might be anticipated that BPO can cause cell damage by a mechanism that does not involve lipid peroxidation.

We have found that BPO is not readily metabolized by mitochondrial glutathione peroxidase and therefore may not

be expected to alter mitochondrial function in the manner ascribed to other peroxidic prooxidant promoters. Herein, we report the effects of BPO on mitochondrial electron and energy transfer and on the morphology of mitochondria.

MATERIALS AND METHODS

Chemicals. Benzoyl peroxide, N-tert-butyl-alpha-phenylnitrone (PBN), and sodium cyanide were purchased from Aldrich. ADP, antimycin A, BSA, catalase, carbonyl cyanide m-chlorophenylhydrazone (CCCP), beta-hydroxybutyrate (BHB, sodium salt), succinate (sodium salt), and superoxide dismutase (SOD) were obtained from Sigma. N,N,N',N'-Tetramethyl-p-phenylenediamine (TMPD) was from Pfaltz and Bauer. 2,4-Dinitrophenol (DNP) was purchased from Matheson, Cole and Bell. Dimethyl-1-pyrroline-N-oxide (DMPO) was obtained from Sigma and purified by diluting with distilled, deionized water, stirring with decolorizing carbon for 5 minutes and then filtering. All other chemicals were commercial samples of high purity and used as supplied. All solutions were made in distilled, deionized water. BPO solutions were prepared in acetonitrile. Antimycin A, CCCP, and TMPD were all used as methanolic solutions.

Preparation of Rat Liver Mitochondria. Adult male Sprague-Dawley rats (300-400 g) were fasted overnight and sacrificed by decapitation. Liver mitochondria were isolated by differential centrifugation (20) using 250 mM sucrose, 3.4 mM Tris (pH 7.4), 1 mM EGTA, and 0.15% BSA. Mitochondria were washed in the same medium without EGTA and BSA.

Preparation of Beef Heart Mitochondria. Beef heart mitochondria were isolated by differential centrifugation (21) and suspended in 250 mM sucrose, 10 mM Tris-Cl (pH 7.8), 1 mM sodium succinate, and 2 mM EDTA.

Preparation of Beef Heart Submitochondrial Particles. After storing the beef heart mitochondria at -20°C for one week, the mitochondria were thawed and submitochondrial particles were prepared by sonication followed by differential centrifugation (22) using 100 mM sodium phosphate buffer (pH 7.4).

Determination of Protein Concentration. The protein concentrations of the mitochondrial and submitochondrial suspensions were determined by the method of Bradford (23) using BSA as a standard.

Measurement of Mitochondrial Respiration. Mitochondrial respiration was determined polarographically using a Clark-type electrode. Mitochondria (1.4-5.0 mg of protein) were added to 3.0 ml (final volume) of medium saturated with air at 30°C and containing 80 mM NaCl, 10 mM MgCl_2 , 4.5 mM sodium phosphate and 5 mM Tris, at a final pH of 7.4. Either 5 mM BHB or 5 mM sodium succinate was used to initiate state 4 respiration. State 3 respiration was initiated by adding 900 nmoles of ADP. Either CCCP (1.6 μM final concentration) or DNP (120 μM final concentration) were used as uncouplers of oxidative phosphorylation.

Difference Spectroscopy. The redox states of the mitochondrial cytochromes were determined in 1 cm cells on a Varian Cary 219 uv-visible spectrophotometer in a manner similar to that described by Chance and Williams (24). The medium used here was the same as that described above for the respiration studies. The reference cell contained oxidized submitochondrial particles (2 mg of protein - no substrate) while the sample cell contained respiring submitochondrial particles (2 mg of protein and 10 mM sodium succinate, final concentration) treated with either antimycin A (2.7 μ M, final concentration) or BPO (13 μ M, final concentration).

Spin Trapping With DMPO in Respiring SMP.

Submitochondrial particles (2 mg of protein), cytochrome c (5.5 nmoles/mg SMP protein) and sodium succinate (10 mM, final concentration) were incubated at room temperature for one minute with DMPO (100 mM, final concentration) in sodium phosphate buffer (33 mM, final concentration, pH 7.4) prior to the addition of either antimycin A (1.6 μ M) or BPO (105 μ M, final concentration). After a one minute incubation in the presence of BPO, the suspension was put in a 17 mm flat cell and degassed by vacuum. The flat cell was then mounted in the TM₁₁₀ wide-bore cavity at X-band frequency. In experiments involving protective enzymes, these were added to the reaction mixture prior to the

addition of the oxidant (final concentrations shown in Fig. 5).

Spin Trapping With DMPO in Respiring Mitochondria.

Uncoupled rat liver mitochondria (8 mg protein) were incubated at room temperature for one minute with DMPO (50 mM, final concentration) in a medium containing 80 mM NaCl, 10 mM MgCl₂, 4.5 mM sodium phosphate, 5 mM Tris, at a final pH of 7.4. Sodium succinate (40 mM) was added to initiate state 4 respiration. BPO (160 uM, final concentration) was added to inhibit respiration. After a one minute incubation in the presence of BPO, the sample was added to a flat cell and treated as described previously.

Spin Trapping With DMPO in Non-Respiring Mitochondria.

Coupled rat liver mitochondria (10 mg protein) were incubated at room temperature for one minute with DMPO (50 mM, final concentration) in a medium containing 0.25 M sucrose and 3.4 mM Tris at a final pH of 7.4. BPO (2 mM, final concentration) was added, and the suspension was incubated for one minute. The sample was then added to a flat cell and treated as described previously.

Spin Trapping With PBN in Non-Respiring Mitochondria.

Coupled rat liver mitochondria (10 mg protein) were incubated at room temperature for one minute with PBN (50 mM, final concentration) in a medium containing 150 mM NaCl and 20 mM Tris at a final pH of 7.4. Either BPO or DMVN (final concentrations given in Fig. 6) was then added, and

the suspension was incubated at 37°C for 5 minutes. The sample was then added to a flat cell and treated as described previously.

HPLC Analysis of Organic Extracts From Mitochondria Treated With BPO. Mitochondria (10 mg protein) were added to 5.0 ml (final volume) of medium saturated with air at 25°C and containing 80 mM NaCl, 10 mM MgCl₂, 4.5 mM sodium phosphate and 5 mM Tris, all at a final pH of 7.4. BHB (15 mM, final concentration) was used to initiate state 4 respiration. State 3 respiration was initiated by adding 0.45 umoles of ADP. Respiration was inhibited by the addition of BPO (0.24 mM, final concentration). Aliquots (1.0 ml) were removed from the suspension at 1, 3, 5, and 7 minutes after the addition of BPO and extracted via Folch extraction (25) using 5.0 ml of 2:1 chloroform/methanol. 2.0 ml of the organic layer was evaporated to dryness on a rotary evaporator. The residue was dissolved in 1.0 ml of 80% hexane/20% dichloromethane and filtered through a Xydex LID/X filter syringe. BPO "blanks" were prepared by adding BPO (0.24 mM, final concentration) to 5.0 ml buffer (see above), removing two 1.0 ml aliquots and extracting in the same manner as described above. The samples were analyzed on a Hewlett Packard 1090 HPLC using a Dupont Zorbax Cyano column (4.6 mm X 25 cm) and a solvent system of 80% hexane/19% dichloromethane/1% n-propanol at a flow rate of 1.0 ml/min.

GC/MS Analysis of Organic Extracts From Mitochondria Treated With BPO. The aqueous layer resulting from Folch extraction of BPO-treated mitochondria (see previous section) was treated with 1 drop of concentrated hydrochloric acid and extracted with 5.0 ml chloroform. The organic layer was evaporated to dryness on a rotary evaporator. The residue was dissolved in 0.5 ml chloroform and filtered through a Xydex LID/X filter syringe. The samples were analyzed on a Hewlett Packard 5890 GC/MS using a DB-17 column (0.179 mm X 20 m).

Assay of Glutathione Peroxidase Activity. The glutathione peroxidase assay was performed using the coupled assay described by Gunzler and Flohe (26). BPO, TBHP and hydrogen peroxide were compared as substrates (final concentrations given in Table IV).

Mitochondrial Swelling. Coupled rat liver mitochondria (ave. RCR = 4.5) were used for all swelling experiments. Mitochondrial suspensions (20 ul - 40 ul) containing approximately 0.5 mg protein were diluted with 3.0 ml suspension buffer (150 mM NaCl, 20 mM Tris, pH 7.4) to give an initial absorbance of 0.6 to 0.8 at 700 nm. BPO was then added to initiate swelling. Swelling was monitored by following the decrease in turbidity of a dilute mitochondrial suspension at 700 nm on a Cary 219 uv-visible spectrophotometer in 1 cm cells at room temperature. This method was similar to that described by

Bhuvaneswaran and Dakshinamurti (27). In experiments involving either protective enzymes, radical scavengers or sulfhydryl reagents, these agents were added to the mitochondrial suspension prior to the addition of BPO (final concentrations shown in Figs. 8, 11 and 12).

Measurement of Lipid Peroxidation by the TBA Test.

Peroxidation of mitochondrial membrane lipids was assessed by the spectrophotometric determination of TBA-reactive products according to the procedure described by Aust (25). Coupled rat liver mitochondria (6 mg protein) were added to 6 ml (final volume) of medium saturated with air at 25°C and containing 150 mM NaCl at a final pH of 7.4. A 1.0 ml aliquot was removed and added to 2.0 ml of stock TBA-TCA-HCl reagent (15% w/v TCA, 0.375% w/v TBA, 0.25 N HCl). Either BPO, TBHP or a mixture of one of these oxidants with FeSO₄ was added to the remaining mitochondria (final concentrations given in Fig. 9). Aliquots (1.0 ml) were removed at 5, 10, 15, and 30 minutes following the addition of the initiator and added to stock TBA-TCA-HCl reagent. The samples were placed in a boiling water bath for 15 min., cooled and centrifuged to remove the precipitate formed during boiling. The absorbance of the samples was measured at 532 nm on a Hewlett Packard UV-visible spectrophotometer against a blank that contained all the reagents but no mitochondria.

RESULTS AND DISCUSSION

BPO Inhibition of Mitochondrial NADH Oxidase and Succinoxidase. Fig. 1 is a composite of several experiments showing the effects of varying concentrations of BPO on mitochondrial beta-hydroxybutyrate oxidase (Fig. 1A) and succinoxidase (Fig. 1B) activities. The addition of BPO during state 3 respiration (ADP-limiting) results in rapid inhibition of oxygen uptake. The uncoupling agent dinitrophenol (DNP) results in a partial release of the inhibition of state 3 respiration. This effect is dependent upon the concentration of BPO; at a final concentration of 15 μ M BPO, DNP has little effect on the rate of respiration while at 7.4 μ M BPO, the DNP-stimulated rate is similar to that seen in the absence of BPO. Similar results are observed with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which is an even more potent uncoupling agent than DNP. Using CCCP, the effects of BPO on uncoupler-stimulated respiration are shown in comparison to those of the classical chain inhibitor antimycin A and the ATPase inhibitor oligomycin A (Table I). When antimycin A is added to mitochondria at concentrations sufficient to cause >90% inhibition of mitochondrial state 3 respiration, little to no stimulation of oxygen uptake is observed upon the addition of CCCP (Table I). When oligomycin A is added to mitochondria at concentrations sufficient to cause 80% inhibition of mitochondrial state 3

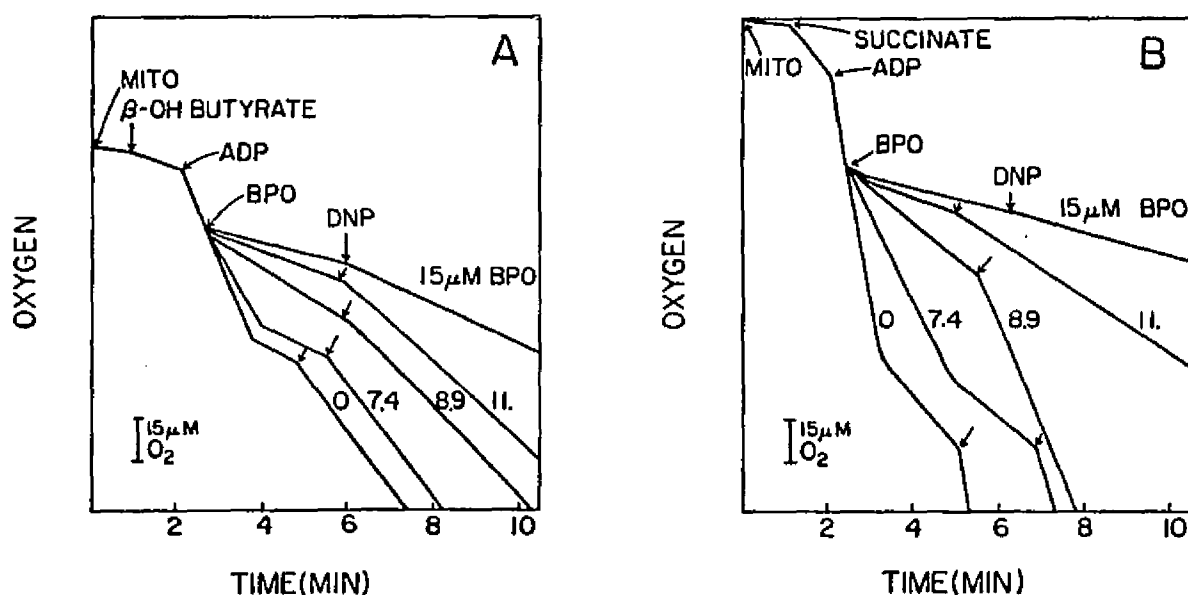


Figure 1: Titration of BHB-respiring mitochondria (Panel A) and succinate-respiring mitochondria (Panel B) with BPO. The composite curves consist of a series of superimposed records of individual experiments in which the final concentration of BPO was varied from 0 to 15 μM (actual concentrations shown in figure). All other concentrations were maintained constant. Mitochondria (2.0 mg of protein) were added to 3.00 ml (final volume) of buffer (80 mM NaCl, 10 mM MgCl_2 , 4.5 mM sodium phosphate, 5 mM Tris, pH 7.4) and the oxygen uptake was measured with a Clark-type oxygen electrode at 30°C. BHB was used to stimulate state 4 respiration in the study of NADH-linked respiration while sodium succinate was used in the study of FADH_2 -linked respiration. ADP was used to stimulate state 3 respiration. DNP was used to test its effect in releasing the respiratory inhibition caused by BPO. The point of addition of these compounds is indicated in the figure by arrows. The final concentrations of the additives were as follows: BHB, 5 mM; sodium succinate, 5 mM; ADP, 300 μM ; DNP, 120 μM .

Table I: Release of BPO Inhibition of Mitochondrial Respiration by CCCP.^a

Inhibitor	nmoles of O per min per mg protein				Difference in % Inhibn (State 3 - CCCP Uncoupled)	% Stimulation of Respiration by CCCP over Corresponding State 3 Value
	+ADP	%Inhibn	+CCCP	%Inhibn		
None	69±6		71±3			3
BPO (13 μ M)	6.5±.3	91	27±3	62	29	315
BPO (20 μ M)	4.6±.3	93	8.4±.5	88	5	83
BPO (26 μ M)	4.8±1.9	93	7.2±.9	90	3	50
Anti A (48 nM)	9.5±3.3	86	22±11	69	17	132
Anti A (64 nM)	5.6±.1	92	7.3±.5	90	2	30
Anti A (128 nM)	5.4±1.0	92	5.6±1.3	92	0	4
Olig A (1.4 μ M)	14±1	80	78±8	-7	87	457

^aMitochondria (2.4 mg of protein) were suspended in 3.00 ml (final volume) of buffer (80 mM NaCl, 10 mM MgCl₂, 4.5 mM Na₂HPO₄, 5 mM Tris, pH 7.4) and the oxygen uptake was measured with a Clark-type oxygen electrode at 30°C. The final concentrations of the additives were as follows: BHB, 5 mM; ADP, 300 μ M; CCCP, 1.6 μ M. The abbreviations Anti A and Olig A refer to antimycin A and oligomycin A respectively. The control values are an average of four determinations \pm standard deviation while the values for the inhibitors are an average of two determinations \pm average deviation. The negative value given for % inhibition by oligomycin A in the presence of CCCP refers to stimulation of oxygen uptake to a greater rate than the control value.

respiration, complete reversal of inhibition is observed upon the addition of CCCP (Table I). At concentrations that cause >90% inhibition of state 3 respiration, the effect of BPO is found to be intermediate between that of antimycin A and oligomycin A. These data indicate that BPO interacts with components of both the mitochondrial electron-transport chain and of the energy-producing assemblies.

As shown in Fig. 2, the inhibition of state 3 mitochondrial respiration by BPO is concentration dependent for both NADH-linked respiration (open symbols) and FADH₂-linked respiration (closed symbols). The concentration of BPO necessary to suppress respiration to 50% of uninhibited controls (IC₅₀) is essentially equal for inhibition of both NADH- and FADH₂-linked respiration by BPO. The similarity of these IC₅₀ values suggests that BPO inhibits both NADH-oxidase and succinoxidase to a similar extent or that inhibition occurs at a site distinct from these complexes. Thus, inhibition may occur at or on the oxidase side of coenzyme Q (Fig. 3).

Reversal of BPO Inhibition by TMPD. When TMPD is added to respiring submitochondrial particles (SMP) inhibited by either antimycin A or BPO, an immediate stimulation of respiration is observed (Table II). This observation generally is taken to indicate a respiratory block on the substrate side of ferricytochrome c, as TMPD

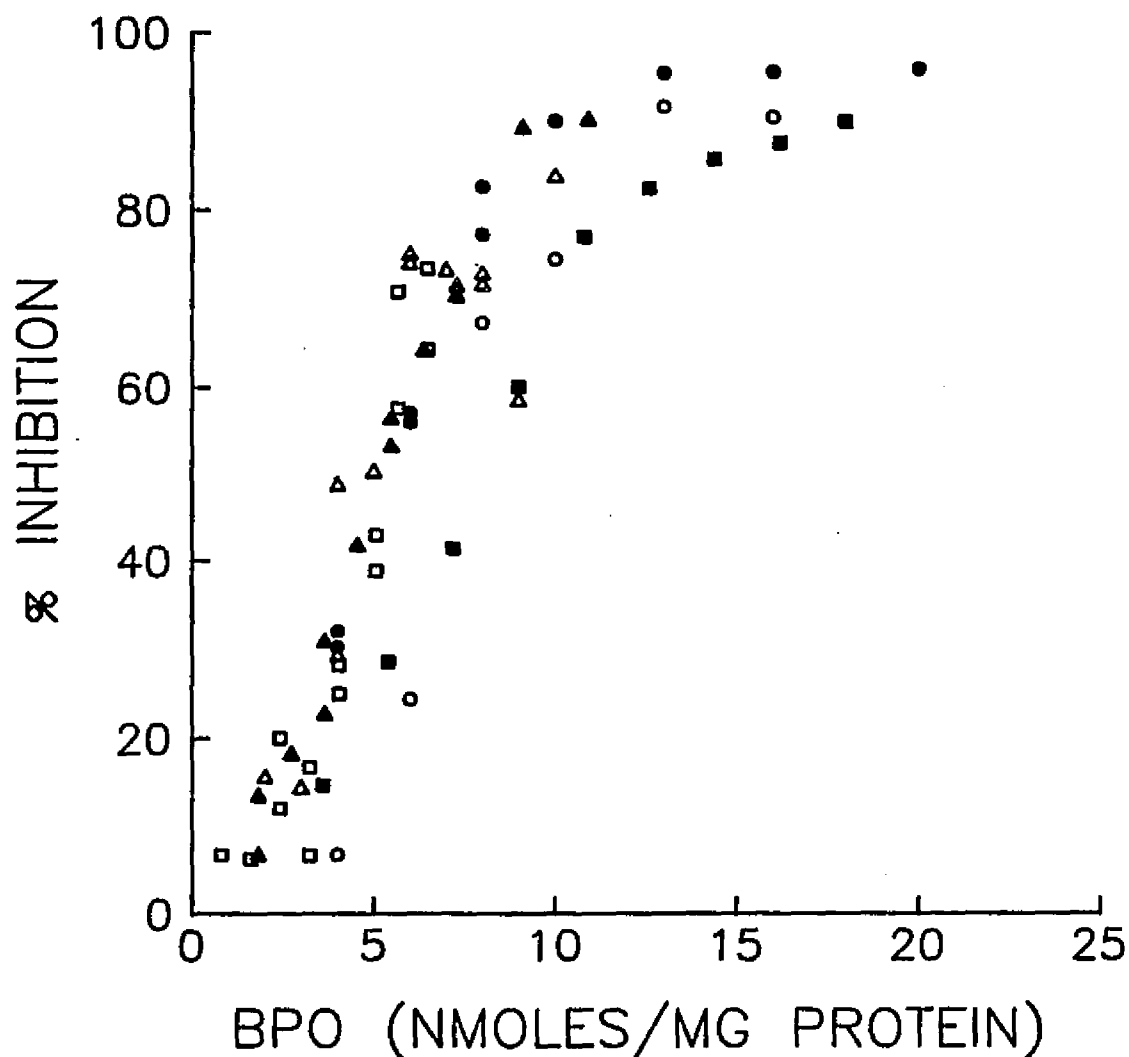


Figure 2: Inhibition of NADH- and FADH₂-linked respiration by BPO as a function of the concentration of mitochondrial protein. Different symbols indicate different mitochondrial preparations. The open symbols are for inhibition of NADH-linked respiration while the closed symbols are for inhibition of FADH₂-linked respiration. The values for % inhibition are calculated by dividing the inhibited rate of respiration by the uninhibited rate of respiration (state 3 rate). Protein concentration ranged between 0.5 and 1.7 mg per ml. Experimental conditions as in Figure 1. IC₅₀ values for the two curves were determined by nonlinear regression analysis using a logarithmic algorithm.

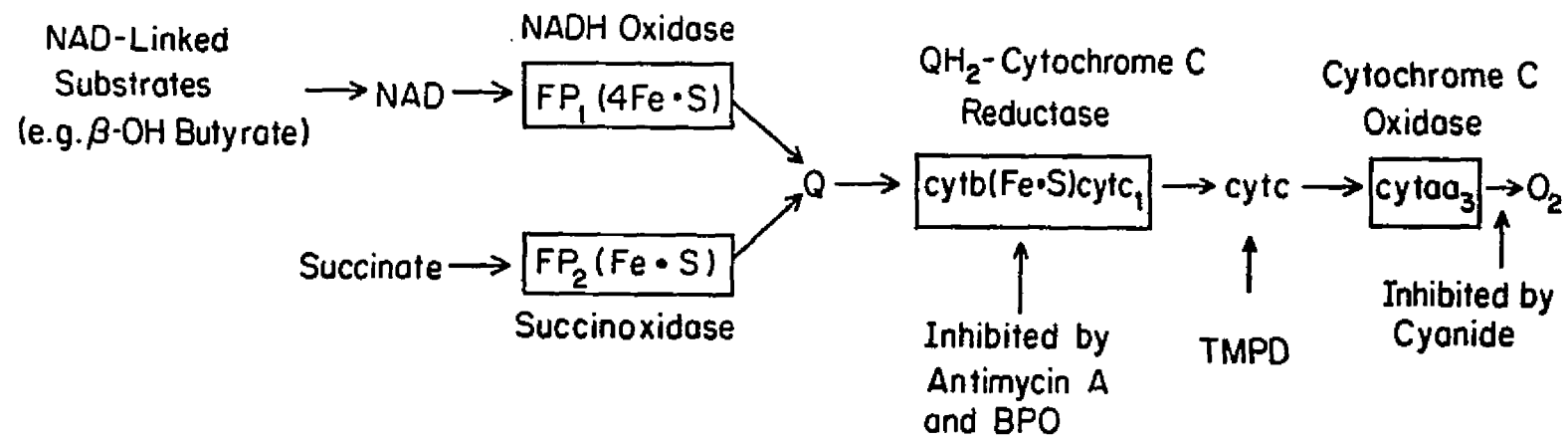


Figure 3: The mitochondrial electron transport chain.

Table II: Effect of the Electron Shunt TMPD on the Rate of Submitochondrial Particle Respiration During Inhibition Induced by Either Antimycin A or Benzoyl Peroxide.^a

Inhibitor	nmoles of O per min per mg protein				% Stimulation of Respiration by TMPD over Inhibited Value	% Inhibition of Respiration by NaCN over TMPD Value
	+Succinate	+Inhib.	+TMPD	+NaCN		
None	89±6		105±8	3.5±2.0		
BPO	76±1	4.4±.5	42±1	3.4±0.3	855	92
Anti A	74±4	4.6±.4	46±16	2.8±0.6	900	94

^aSubmitochondrial particles (0.5 mg of protein) were suspended in 3.00 ml (final volume) of buffer (80 mM NaCl, 10 mM MgCl₂, 4.5 mM Na₂HPO₄, 5 mM Tris, pH 7.4) and the oxygen uptake was measured with a Clark-type oxygen electrode at 30°C. The final concentrations of the additives were as follows: sodium succinate, 5 mM; TMPD, 0.3 mM; NaCN, 1 mM; BPO, 6.7 μM; antimycin A, 0.2 μM. Each value is an average of two determinations ± average deviation.

is known to donate electrons to cytochrome c (28). The TMPD-bypassed respiration of either the antimycin A-blocked or BPO-blocked respiratory chain is inhibited by cyanide, an inhibitor of cytochrome oxidase. BPO is a potent oxidant that reacts directly with TMPD (unpublished results). In the SMP system, this reaction may lead to destruction of the BPO with subsequent release of respiratory inhibition. Although this reaction could not be rigorously excluded, the fact that cyanide inhibits TMPD-stimulated oxygen uptake indicates that BPO does not irreversibly damage or inhibit cytochrome oxidase.

Effect of BPO on the Redox States of Mitochondrial Cytochromes. When BPO is added to succinate-respiring SMP, only the major alpha and gamma (Soret) bands of reduced cytochrome b are observed to accumulate in the difference spectrum (reduced minus oxidized) in a manner analogous to that observed in the presence of antimycin A (Fig. 4). This result indicates that BPO inhibits the flow of electrons from the substrate side of cytochrome c. Although antimycin A and BPO both cause an accumulation of reduced cytochrome b, this result does not necessarily mean that they inhibit the electron transport chain at the same site. According to the mechanism of the protonmotive Q cycle (29), antimycin A inhibits reoxidation of cytochrome b-562. BPO may act in a similar manner or it may interact

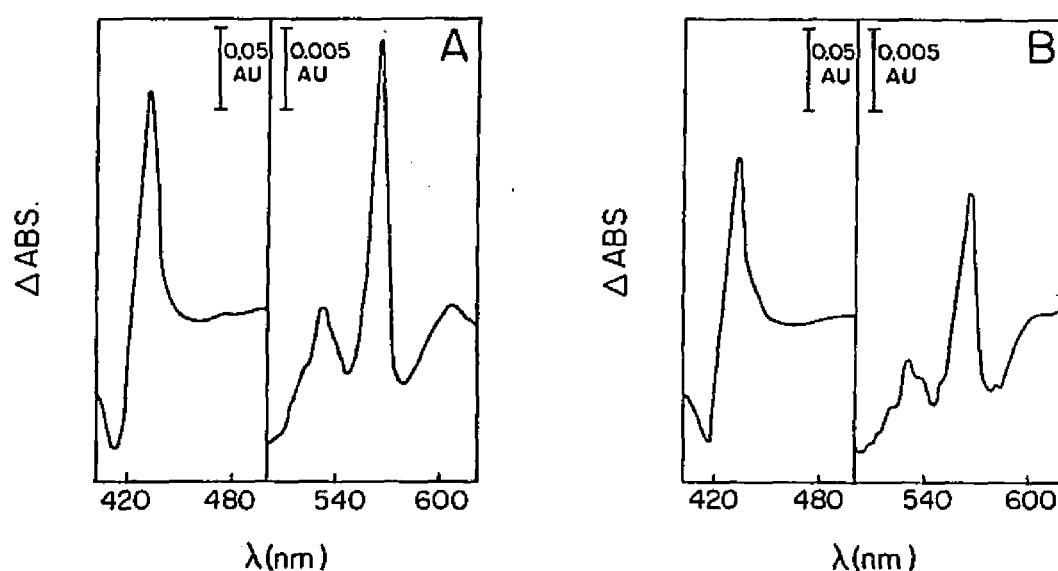


Figure 4: Difference spectrum of respiring submitochondrial particles treated with either antimycin A (Panel A) or BPO (Panel B). Submitochondrial particles (2.0 mg of protein) were added to 1.50 ml (final volume) of buffer (80 mM NaCl, 10 mM MgCl_2 , 4.5 mM sodium phosphate, 5 mM Tris, pH 7.4) in each of two cells. Sodium succinate (10 mM, final concentration) and either antimycin A (2.7 μM , final concentration) or BPO (13 μM , final concentration) were added to the sample cell. The difference spectrum was measured at 25°C on a Cary 219 UV-visible spectrophotometer.

with the iron-sulfur protein that donates electrons to cytochrome c_1 .

Action of Antimycin A and BPO on Radical Production in Submitochondrial Particles. Respiring mitochondria have been shown to produce superoxide radicals via redox cycling of the coenzyme Q semiquinone free radical (30). The production of superoxide radicals is stimulated in the presence of antimycin A (30) and steady state concentrations of $O_2^{\cdot -}$ and H_2O_2 are established (31). Superoxide and hydroxyl radicals can be detected by ESR from respiring beef heart mitochondria treated with antimycin A in the presence of the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (32). We have obtained similar results with respiring SMP using either antimycin A (Fig. 5A) or BPO (Fig. 5D) as a respiratory chain inhibitor.

When antimycin A is added to succinate-respiring SMP in the presence of the spin trap DMPO, an ESR spectrum resulting from the trapping of two radical intermediates is observed (Fig. 5A). The major, four-line spin adduct has hyperfine splitting constants (hfsc) of $a_N = a_H = 1.49$ mT, consistent with a hydroxyl radical spin adduct (33). The hfsc of the other adduct could not be measured due to the weakness of this signal, although the adduct has been tentatively identified as the superoxide radical spin adduct by comparison of the experimental spectrum with a gaussian simulation of both adducts (Fig. 5B). Our

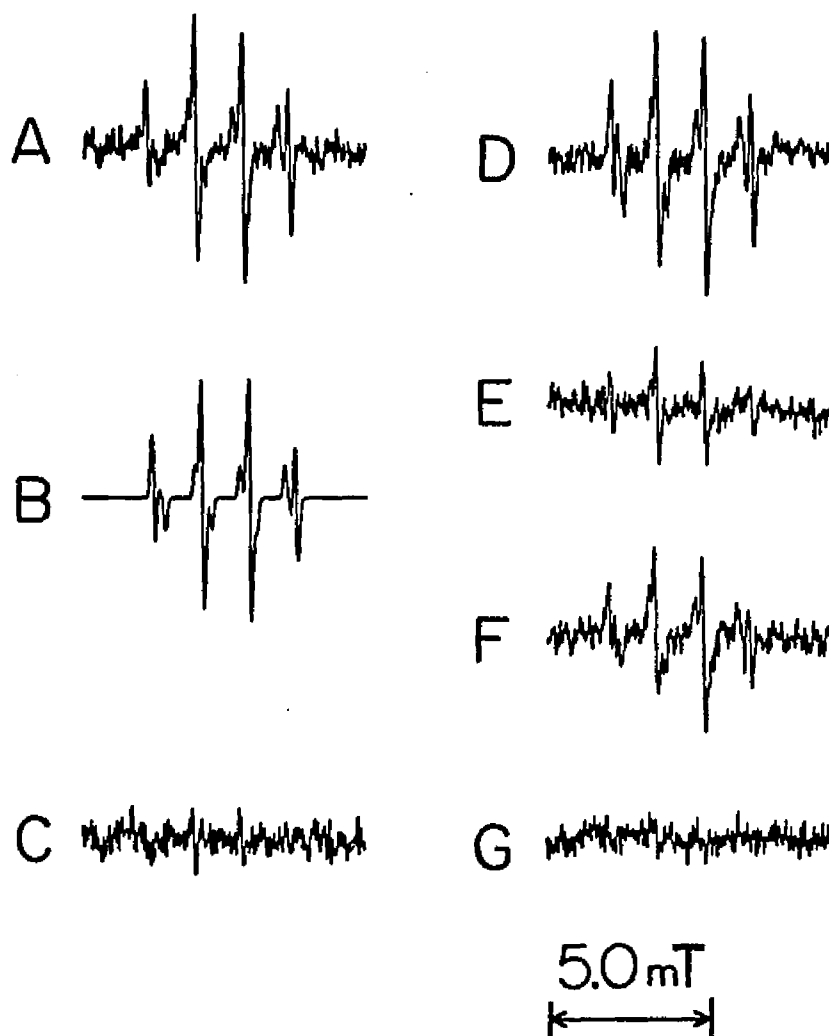


Figure 5: Effect of protective enzymes on BPO-induced radical production in respiring submitochondrial particles. (A) Incubation containing SMP (2.0 mg of protein), 5.5 nmoles cytochrome c per mg protein, 10 mM sodium succinate, 100 mM DMPO, and 1.6 μ M antimycin A in buffer (0.1 M sodium phosphate, chelexed, pH 7.4). (B) Gaussian simulation of the DMPO-OH and DMPO-OOH adducts. (C) Same as in A except that no antimycin A was present. (D) Incubation containing SMP (2.0 mg of protein), 5.5 nmoles cytochrome c per mg protein, 10 mM sodium succinate, 100 mM DMPO, and 105 μ M BPO in buffer (0.1 M sodium phosphate, chelexed, pH 7.4). (E) Same as in D except 100 μ g per ml of SOD was present. (F) Same as in D except 1000 μ g per ml of catalase was present. (G) Same as in D except 100 μ g per ml of SOD and 1000 μ g per ml of catalase were present. These ESR experiments were carried out on an IBM Instruments model ER200-SRC spectrometer equipped with an Aspect 2000 data system. The following instrument conditions were used: microwave power, 20 mW; modulation amplitude, 1.25 G; receiver gain, 2×10^6 ; time constant, 500 ms; sweep width, 90 G; sweep time, 200 s; number of scans, 2.

experimental hfsc values for the hydroxyl adduct (given above) and values for the hfsc of the DMPO-superoxide adduct ($a_N = 1.42$ mT, $a_H = 1.13$ mT, and $a_H = 0.13$ mT) formed by the xanthine/xanthine oxidase system (unpublished results) have been used to generate the simulated spectrum. No significant radical production is observed with respiring SMP in the absence of antimycin A (Fig. 5C). Addition of superoxide dismutase (SOD) to the SMP suspension prior to the addition of BPO results in a reduction in the intensity of both the hydroperoxyl and hydroxyl spin adduct (Fig. 5E). The reduction in the intensity of both the DMPO-OOH and DMPO-OH adducts by SOD suggests that some hydroxyl adduct formation is due to decomposition of the DMPO-OOH adduct (34). Catalase causes attenuation of only the hydroxyl adduct signal (Fig. 5F), indicating that some of the DMPO-OH adduct results from direct trapping of the H_2O_2 -derived hydroxyl radical. Addition of both catalase and SOD results in complete inhibition of both the DMPO-OOH and DMPO-OH signals (Fig. 5G). The fact that oxygen radical production is increased when respiring SMP are treated with BPO in the presence of DMPO is consistent with inhibition of electron flow by BPO at a locus after coenzyme Q in the electron transport chain. Furthermore, BPO does not yield spin-trappable radicals in non-respiring SMP, indicating that the observed radical production in respiring SMP must be related to

electron transport and not to radicals generated via the decomposition of BPO.

Action of BPO on Radical Production in Intact Mitochondria. tert-Butyl hydroperoxide, another free radical initiator, induces the production of spin-trappable radicals in both respiring and non-respiring rat liver mitochondria (16). BPO does not yield spin-trappable radicals when added to intact liver mitochondria (respiring or non-respiring) in the presence of DMPO. This spin trap partitions significantly into the aqueous phase. Thus, radicals generated in the lipid bilayer of the mitochondrial membrane may not be trapped by DMPO. To investigate this possibility, mitochondria have been incubated at 37°C in the presence of PBN, a more lipophilic spin trap, and BPO for 5 minutes. Following this incubation, no ESR spin adducts are observed (Fig. 6A). 2,2'-Azo-bis(2,4-dimethylvaleronitrile) (DMVN) has been used to confirm that radicals can be generated in mitochondria via thermal decomposition of a free radical initiator. When DMVN is substituted for BPO, spin adducts attributable to both a carbon-centered and oxygen-centered adduct are observed (Fig. 6B). The carbon-centered adduct has measured hfsc of $a_N = 1.53$ mT and $a_H = 0.33$ mT while the oxygen-centered adduct has measured hfsc of $a_N = 1.40$ mT and $a_H = 0.20$ mT. The inability of BPO to yield radicals in mitochondria may be due to loss of this.

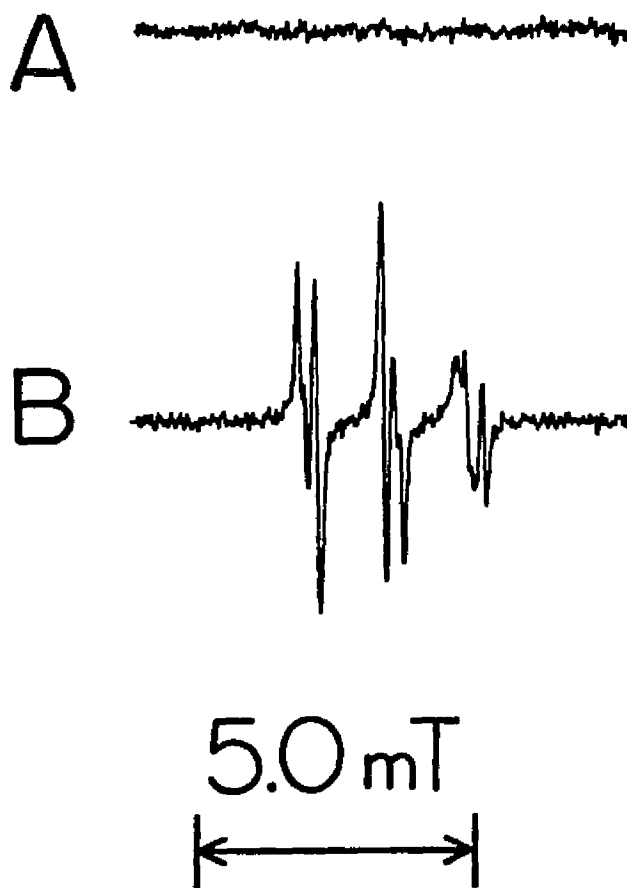


Figure 6: Initiator-induced radical production in non-respiring liver mitochondria. (A) Incubation containing mitochondria (5.0 mg protein), 47 mM PBN, and 9.4 mM BPO in buffer (0.15 M NaCl, 0.02 M Tris, pH 7.4). (B) Same as in A except that 9.4 mM DMVN was present instead of BPO. These ESR experiments were carried out on an IBM Instruments model ER100D spectrometer equipped with an Aspect 2000 data system. The following instrument conditions were used: microwave power, 20 mW; modulation amplitude, 0.63 G; receiver gain, 8×10^5 ; time constant, 200 ms; sweep width, 90 G; sweep time, 200 s; number of scans, 3.

compound via induced decomposition (35,36), which results in low efficiency of radical production in polar solvents.

Fate of BPO During Inhibition of Respiration. Table III shows the amount of BPO recovered by organic extraction after one minute from typical mitochondrial incubations. Essentially all of the BPO disappears in the presence of both respiring and non-respiring mitochondria. With heat-denatured mitochondria, approximately 25% of the BPO still remained after one minute of incubation. This residual BPO disappeared completely over the next two minutes. Even though heat denaturation of the mitochondrial enzymes slows BPO disappearance, the disappearance is still accelerated; in the absence of mitochondria, no loss of BPO is observed over longer time periods. Despite the rapid loss of HPLC-detectable BPO in the presence of mitochondria, intact or not, no BPO derived-products are observed in organic extracts of mitochondria, suggesting tight binding or sequestration. However, benzoate, a product of the induced decomposition of BPO (35,36), would be found in the aqueous layer following extraction of BPO-treated mitochondria. The presence of benzoate has been probed by treating the aqueous layer with acid, extracting with chloroform and analyzing the extract by GC/MS. By this method, we have determined that benzoate is formed when mitochondria are treated with BPO. Thus, the loss of BPO over time can be

Table III: Amount of BPO Recovered From Buffer
and Mitochondria by Organic Extraction.^a

Type of Incubation	nmoles of BPO Extracted
Buffer	203±2
Respiring Mitochondria	1.3±0.1
Non-Respiring Mitochondria	3.9±0.2
Heat Denatured Mitochondria	49±3

^aMitochondria (10.0 mg of protein) were suspended in 5.0 ml (final volume) of buffer (80 mM NaCl, 10 mM MgCl₂, 4.5 mM Na₂HPO₄, 5 mM Tris, pH 7.4) at 25°C. The final concentrations of the additives were as follows: BHB, 15 mM; ADP, 0.90 mM; BPO, 0.24 mM. BHB and ADP were added in the respiring mitochondria incubation only. Mitochondria were denatured by heating for two minutes in a boiling water bath. Extraction and analysis of samples as described under Methods. Each value is an average of two determinations ± average deviation.

attributed, at least in part, to the conversion of BPO to benzoate.

Effect of BPO on the Turbidity of Mitochondrial Suspensions. Lysis of the mitochondrial membrane is often a consequence of mitochondrial swelling (37). Because other peroxidic compounds are known to cause mitochondrial swelling, the effects of BPO on mitochondrial swelling have been studied.

The addition of BPO to non-respiring rat liver mitochondria results in rapid, large-amplitude swelling. The rate and amplitude of swelling is dependent on the ratio of BPO to mitochondrial protein but is independent of respiratory substrate (Fig. 7). BPO-induced mitochondrial swelling appears to be independent of endogenous calcium, as neither ruthenium red, a calcium channel blocker, nor EGTA, a calcium chelator, altered the rate of swelling when incubated with mitochondria prior to the addition of BPO. These results suggest that the mechanism of BPO-induced swelling is different than that of hydroperoxide-induced swelling, which is dependent on calcium cycling. Hydroperoxide-induced swelling is also dependent upon the ability of the hydroperoxide to serve as a substrate for glutathione peroxidase (13-15). The data in Table IV show that under conditions in which TBHP and H_2O_2 readily promote glutathione peroxidase activity (38), BPO is ineffective. This lack of activity provides further

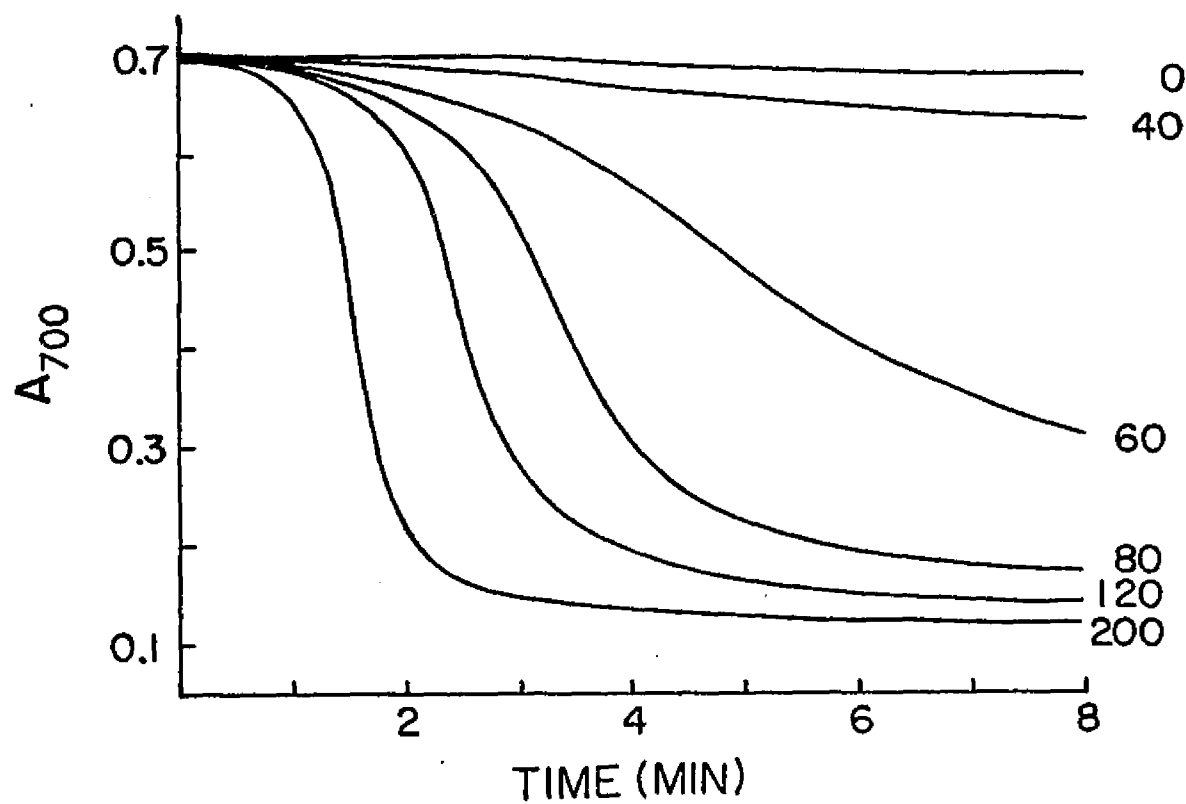


Figure 7: Induction of mitochondrial swelling by BPO. The composite curve consists of a series of superimposed records from individual experiments in which the amount of BPO added was varied. The numbers next to the curves refer to amount of BPO added in units of nmoles BPO/mg mitochondrial protein. Mitochondria (0.50 mg of protein) were added to 3.00 ml buffer (0.15 M NaCl, 0.02 M Tris, pH 7.4). The change in optical density of the mitochondria was monitored at 25°C on a Cary 219 UV-visible spectrophotometer set at 700 nm.

Table IV: Activity of Peroxides as Substrates for Glutathione Peroxidase.^a

Peroxide	NADPH Oxidation in umole/l/sec				Relative Activity
	NADPH	NADPH + GSH + GSSG Reductase	NADPH + GSH + GSSG Reductase + GSH Peroxidase	Ratio of Catalyzed to Uncatalyzed	
None	0.05±0.00 ₅	0.03±0.00 ₂	0.09±0.02	3	—
0.1 mM H ₂ O ₂	—	0.06±0.00 ₁	1.18±0.03	20	100
1.0 mM TBHP	—	0.10±0.00 ₁	1.36±0.03	14	70
0.1 mM BPO	0.09±0.01	0.34±0.09 ^b	0.50±0.08 ^b	1.5	7.5
0.2 mM BPO	0.19±0.02	1.08±0.06 ^b	1.08±0.18 ^b	1.0	5.0

^aThe assay was carried out in phosphate buffer (1 mM EDTA, 0.1 M sodium phosphate, pH 7.0) at 37°C. The oxidation of NADPH was monitored at 340 nm on a HP UV-visible spectrophotometer. The final concentrations of the reactants were as follows: NADPH, 0.15 mM; GSH, 1.0 mM; GSSG reductase, 0.24 units per ml; GSH peroxidase, 0.10 unit per ml. Each value is an average of three determinations ± standard deviation.

^bThese are initial rates of NADPH oxidation, as the change in absorbance at 340 nm in these experiments was non-linear with time.

evidence that BPO induces swelling by a different mechanism than does TBHP.

Determination of the Role of Free Radicals in the Mechanism of BPO-Induced Swelling. Glutathione peroxidase, catalase and SOD do not cause any significant inhibition of BPO-induced mitochondrial swelling. Thus, either swelling is not oxygen radical-dependent or the site of production and reaction of these oxyradical species is inaccessible to the protective enzymes. The radical scavenger sodium benzoate also has no effect on BPO-induced swelling (Fig. 8), implying that free hydroxyl radicals are not involved in the process. GSH and alpha-tocopherol (a-T) significantly inhibit BPO-induced mitochondrial swelling (Fig. 8). We have studied the reaction between BPO and GSH and have found that these compounds react rapidly to cause the oxidation of GSH to GSSG (unpublished results). BPO also reacts directly with a-T (39,40). Thus, the inhibition of mitochondrial swelling with GSH and a-T may be due to the destruction of BPO via direct reaction with these compounds. As described previously, BPO does not induce the production of spin-trappable radicals in whole mitochondria as determined by ESR (Fig. 6A). Surprisingly, the data in Fig. 9 show that BPO does induce the production of TBA reactive compounds (41), although the concentration of products is low when compared to either a TBHP/Fe²⁺ or BPO/Fe²⁺ initiating system. Ferrous ions enhance the

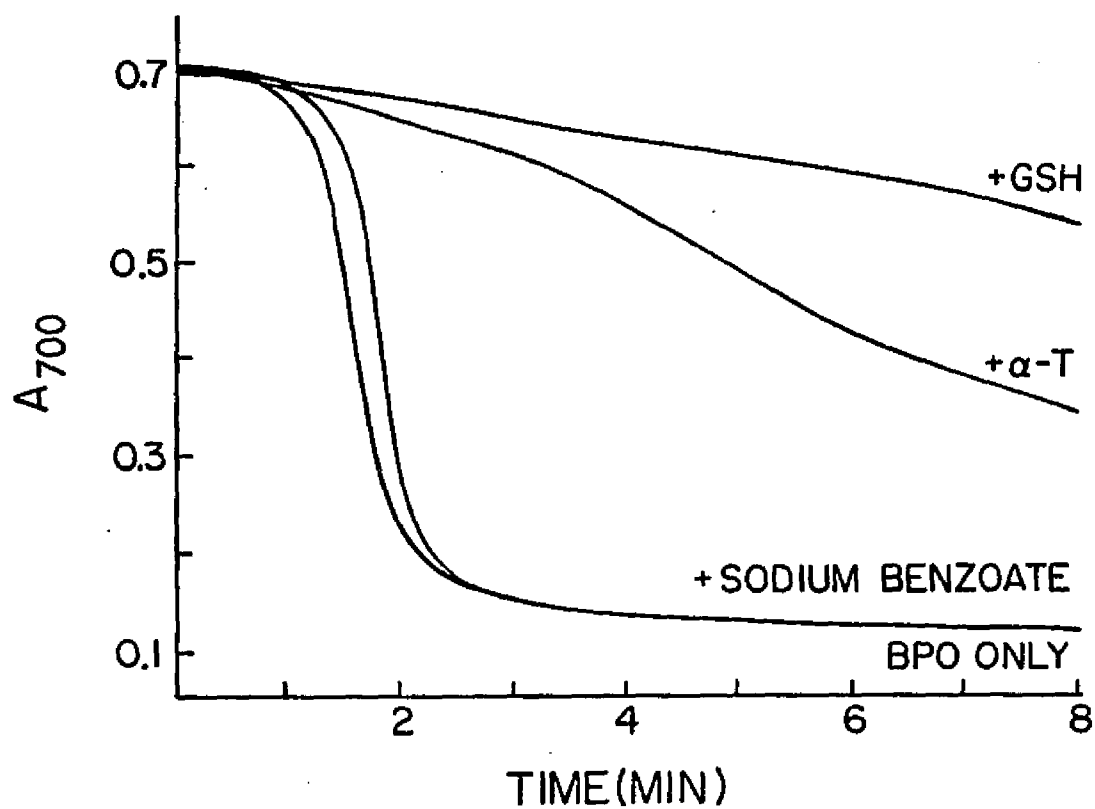


Figure 8: Effect of Radical Scavengers on BPO-Induced Mitochondrial Swelling. Experimental conditions as in Figure 7. Scavengers were incubated with mitochondria for 2 min prior to addition of BPO. The final concentrations of the additives were as follows: BPO, 33 μ M; GSH, 100 μ M; α -T, 100 μ M; sodium benzoate, 100 μ M.

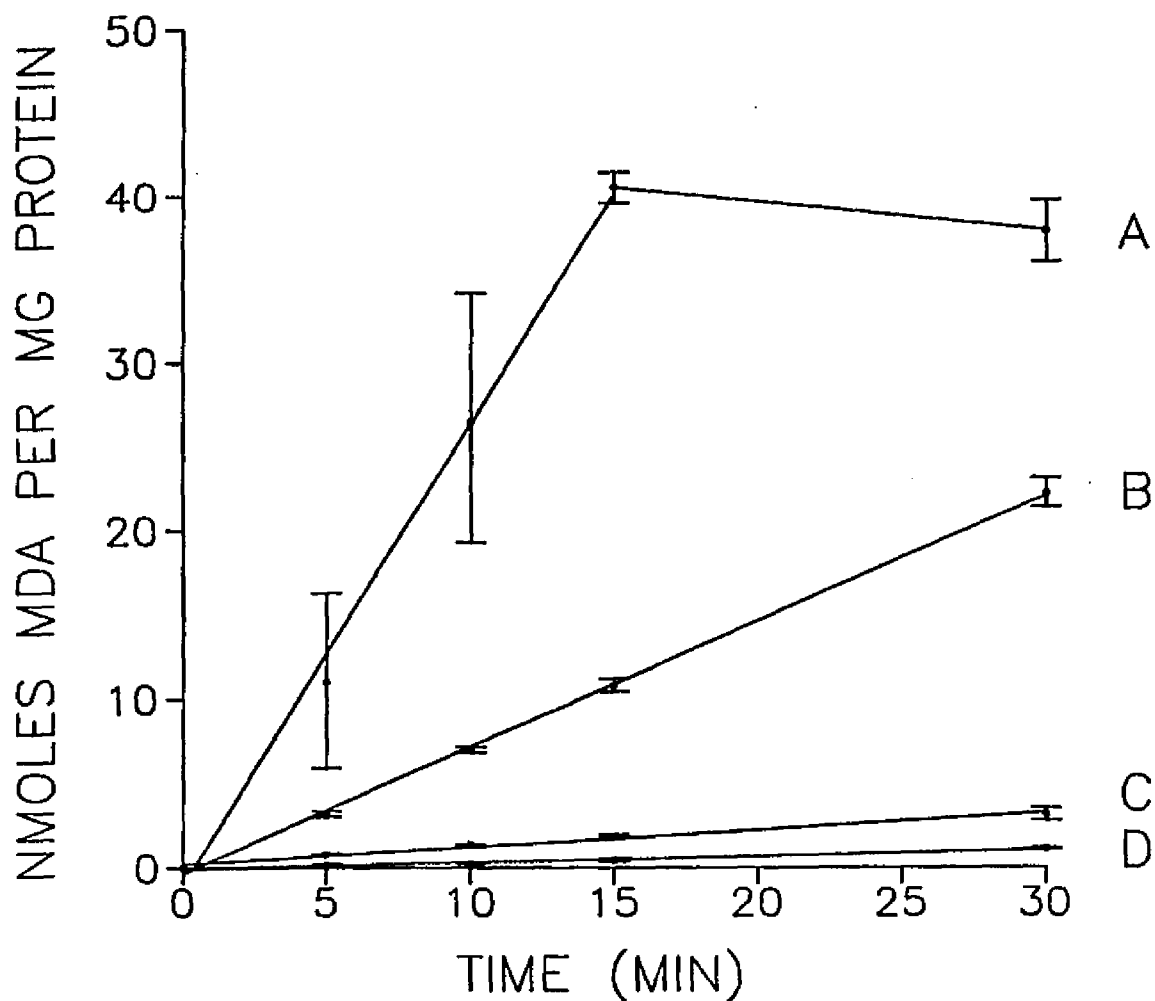


Figure 9: Malondialdehyde Formation in Mitochondria Incubated With Peroxidic Initiating Systems. The initiators were added to 5.0 mg mitochondrial protein in 0.15 M NaCl at 25°C. 1.0 ml aliquots were removed from the suspension and treated in the manner described under Methods. The final concentrations of the initiators were as follows: (A) BPO, 0.2 mM; FeSO₄, 0.8 mM; (B) TBHP, 0.8 mM; FeSO₄, 0.8 mM; (C) BPO, 0.2 mM; (D) TBHP, 0.8 mM. Each value is an average of three determinations \pm average deviation.

ability of BPO to induce mitochondrial lipid peroxidation (Fig. 9). It would be expected that if lipid peroxidation is involved in BPO-induced mitochondrial swelling, then ferrous ions should similarly stimulate swelling. However, when mitochondria are treated with FeSO_4 and BPO, a significant reduction in the rate of BPO-induced swelling is observed (Fig. 10). These results indicate that the swelling observed with BPO alone is not due to lipid peroxidation. Furthermore, we have determined that BPO-induced swelling is independent of molecular oxygen; the rate and magnitude of swelling determined in suspension buffer purged with argon for up to 2 hours prior to the addition of mitochondria and BPO is the same as that found in the presence of O_2 . Therefore, any oxidative reactions that occur when BPO is added to mitochondria probably reflect direct reaction of BPO with mitochondrial components.

Determination of the Role of Mitochondrial Sulfhydryl Groups in the Mechanism of BPO-Induced Swelling. Oxidation of mitochondrial sulfhydryl groups has been implicated in the mechanism by which chlorinated naphthoquinones induce mitochondrial swelling (42,43). To determine whether oxidation of mitochondrial sulfhydryl groups is involved in BPO-induced swelling, the effect of several thiol reagents have been examined (Fig. 11). Addition of either cysteine, DTT, GSH or mercaptoethanol at 200 nmoles thiol/mg protein

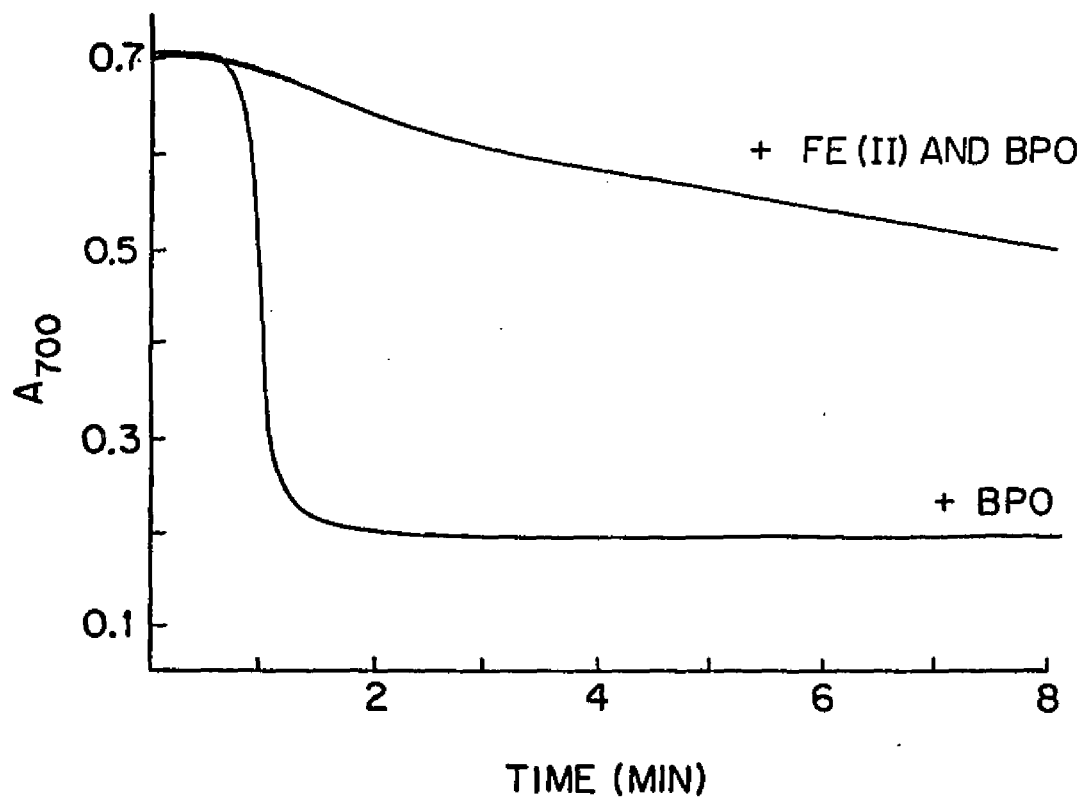


Figure 10: Effect of Ferrous Ions on BPO-Induced Mitochondrial Swelling. Experimental conditions as in Figure 7. FeSO_4 and BPO were added to the mitochondria at the same time. The final concentrations of the additives were as follows: BPO, 39 μM ; FeSO_4 , 133 μM .

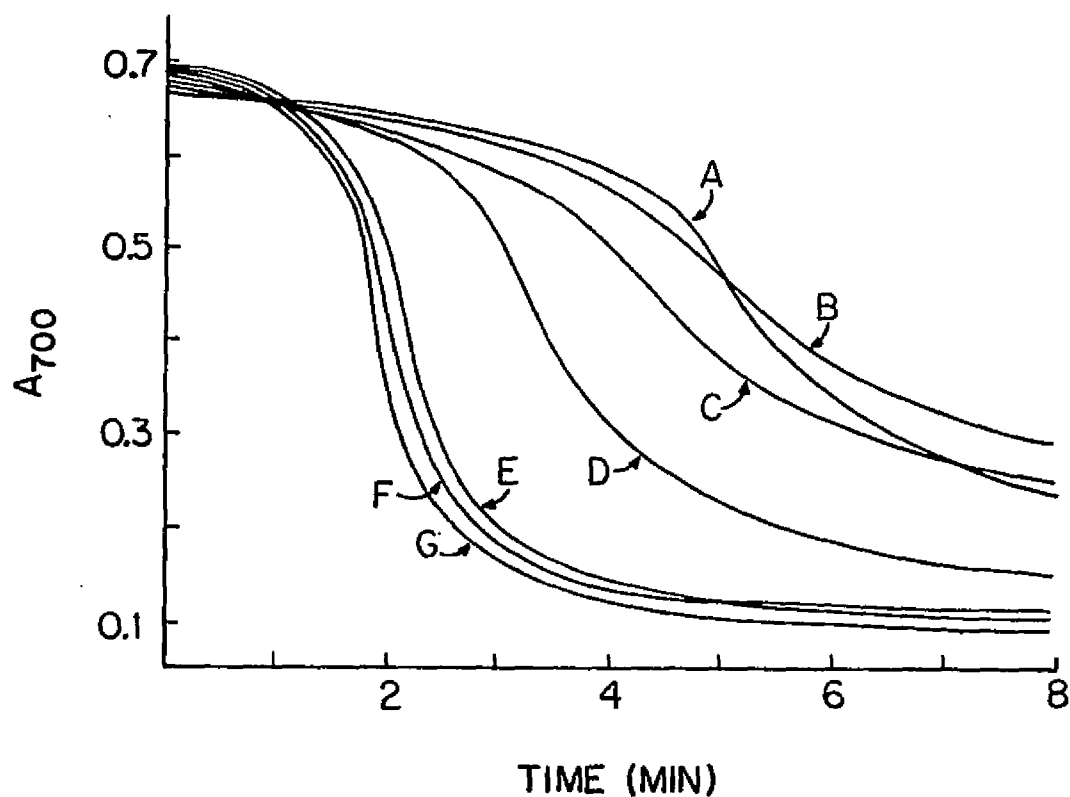


Figure 11: Effect of Thiols on BPO-Induced Mitochondrial Swelling. Experimental conditions as in Figure 7 except that 0.52 mg of mitochondria were used. Thiols were incubated with mitochondria for 2 min. prior to addition of BPO. The final concentration of BPO was held constant at 33 μ M. The final concentrations of the thiols were as follows: (A) cysteine, 33 μ M; (B) DTT, 33 μ M; (C) methionine, 0.32 mM; (D) mercaptoethanol, 33 μ M; (E) methionine, 33 μ M; (F) GSSG, 0.32 mM; (G) no thiol added.

results in significant inhibition of swelling. On a per mole basis, cysteine and GSH are more effective inhibitors than are DTT and mercaptoethanol. A much larger amount of methionine (2000 nmoles/mg protein) is necessary to observe inhibition of swelling. Oxidized glutathione (GSSG) does not protect against BPO-induced mitochondrial swelling, indicating that oxidizable sulfur is necessary to inhibit swelling. Taken as a whole, the inhibition of swelling by thiol reagents suggests that mitochondrial sulfhydryl groups are oxidized during BPO-induced swelling.

More rigorous proof would be direct measurement of mitochondrial sulfhydryl groups. We have attempted this using both Ellman's reagent (44) and NEM (45). Unfortunately, BPO oxidizes the 3-carboxyl-4-nitrophenol sulfide ion that is formed from the reaction between DTNB and reduced thiols and thus, interferes with the Ellman assay (unpublished results). In the NEM method, a water-soluble mitochondrial component that absorbs near the NEM peak interferes with its measurement (unpublished results).

Reagents that derivatize endogenous thiols, DTNB, NEM, PMB and mersalyl, have no significant effect on BPO-induced mitochondrial swelling (Fig. 12), suggesting that oxidation of mitochondrial sulfhydryl groups is not the rate-limiting step in this swelling.

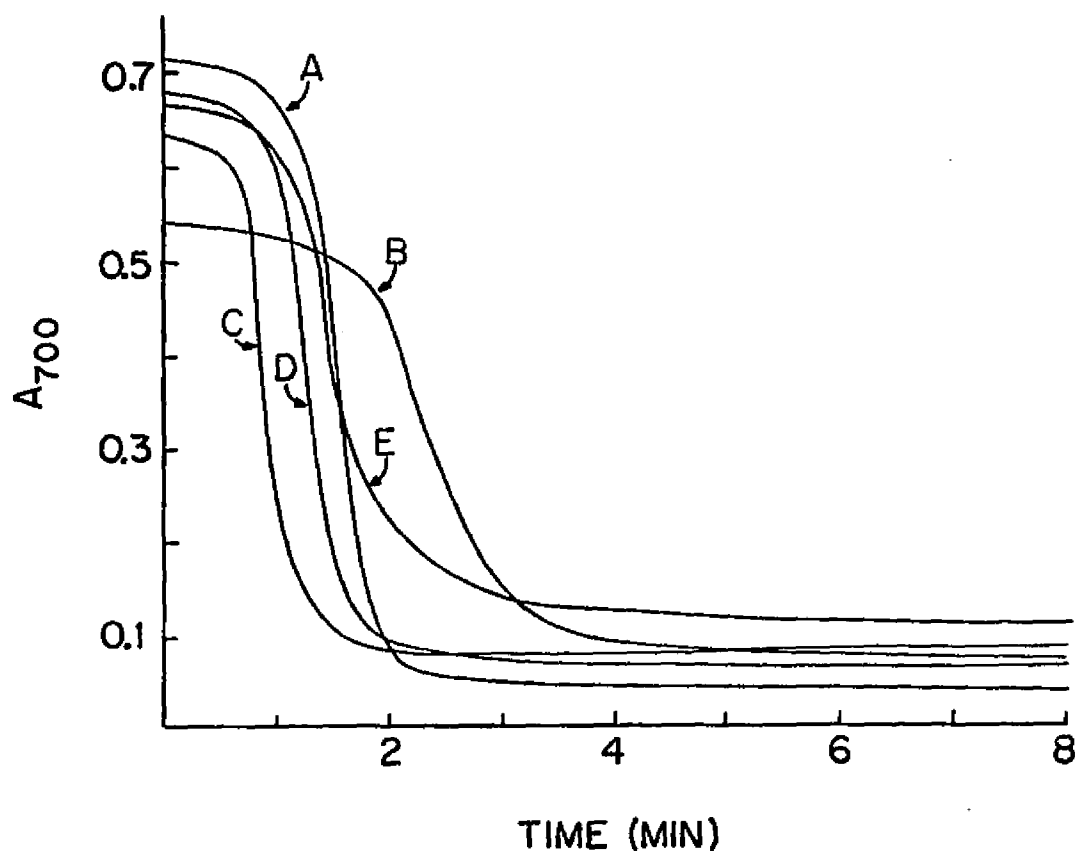


Figure 12: Effect of Sulfhydryl Group Reagents on BPO-Induced Mitochondrial Swelling. Experimental conditions as in Figure 7. All sulfhydryl group reagents were incubated with mitochondria for 8 min. prior to the addition of BPO except for PMB which was incubated for 2 min. There was no incubation period prior to addition of BPO in the control experiment where no sulfhydryl group reagent was used. The final concentration of BPO was held constant at 33 μ M. The final concentrations of the sulfhydryl group reagents were as follows: (A) no sulfhydryl group reagent added; (B) DTNB, 81 μ M; (C) NEM, 33 μ M; (D) PMB, 17 μ M; (E) mersalyl, 81 μ M.

Determination of the Role of Monovalent Cations and Ionic Strength in the Mechanism of BPO-Induced Swelling.

Very slow, low-amplitude swelling is observed when the mitochondrial experiments are conducted in an isotonic sucrose medium (Fig. 13). The rate and amplitude of swelling increase when mitochondria are suspended in a solution containing either Na^+ or Cs^+ and then treated with BPO (Fig. 13), indicating that the presence of monovalent cations are required for rapid, large-amplitude swelling. The fact that swelling is similar in the presence of either Na^+ or Cs^+ shows that BPO-induced mitochondrial swelling is not monovalent cation specific. The osmotic nature of this swelling has been further investigated as a function of varying ionic strength (Fig. 14). When the ionic strength of the suspension medium is increased from 0.15 M NaCl to 0.17 M NaCl, an increase in the amplitude of BPO-dependent swelling is observed. A further increase in amplitude and decrease in the induction period prior to the onset of swelling are seen when the mitochondria are suspended in 0.15 M NaCl, 20 mM Tris (pH 7.4). Taken as a whole, the osmoregulation studies suggest that BPO initiates passive osmotic swelling in mitochondria that is dependent on the presence of monovalent cations and on the ionic strength of the swelling medium.

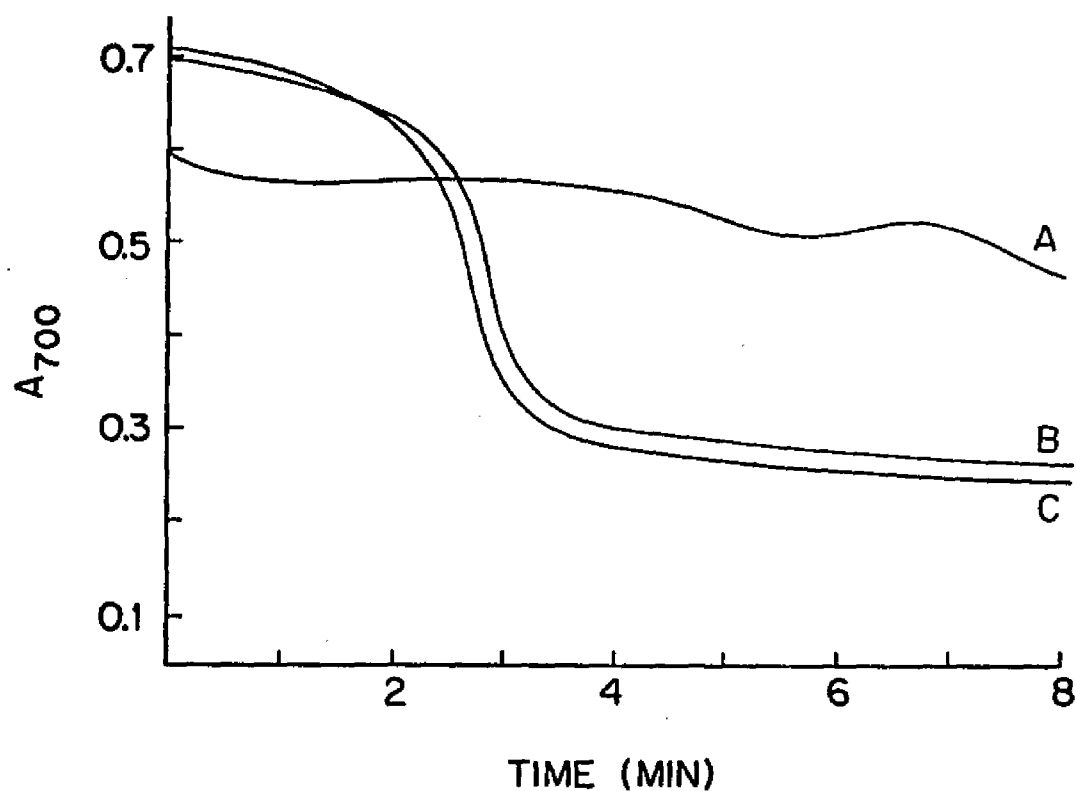


Figure 13: Effect of Monovalent Cations in the Suspension Medium on BPO-Induced Mitochondrial Swelling. Experimental conditions as in Figure 7 except that mitochondria (0.52 mg of protein) were added to either (A) 3.00 ml sucrose solution (0.25 M, unbuffered), (B) 3.00 ml NaCl solution (0.15 M, unbuffered) or (C) 3.00 ml CsCl solution (0.15 M, unbuffered). The final concentration of BPO was held constant at 33 μ M.

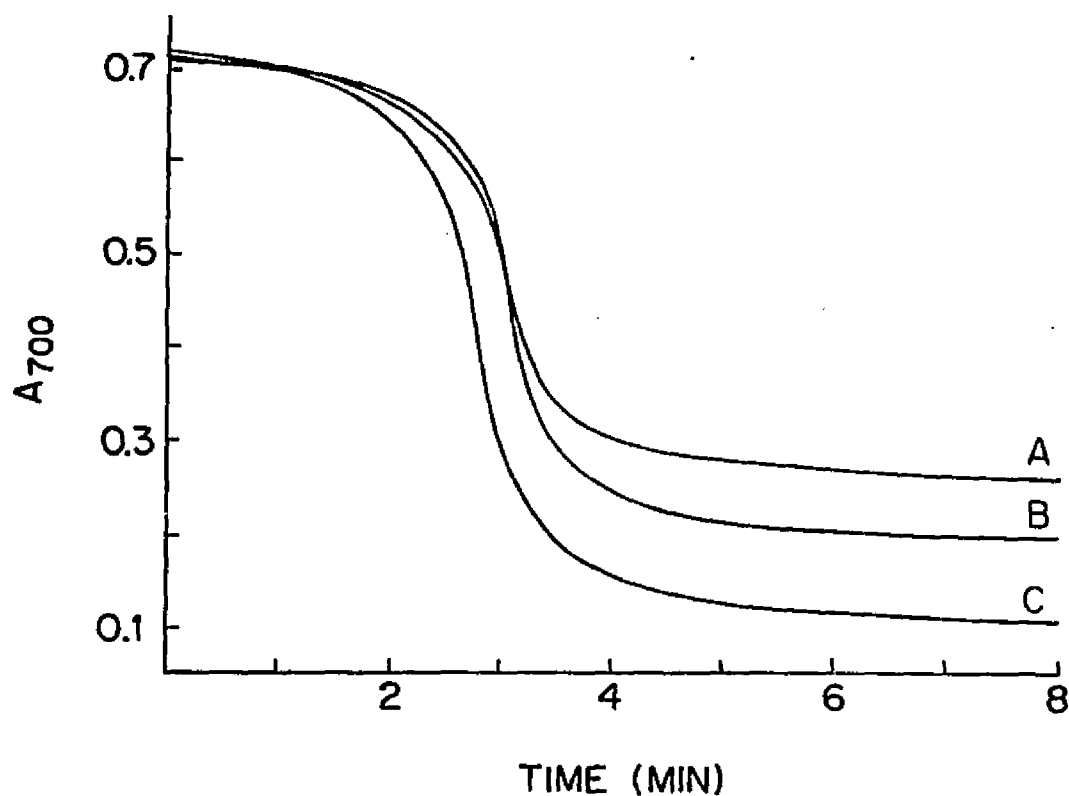


Figure 14: Effect of Ionic Strength on BPO-Induced Mitochondrial Swelling. Experimental conditions as in Figure 7 except that mitochondria (0.52 mg protein) were added to either (A) 3.00 ml NaCl solution (0.15 M, unbuffered), (B) 3.00 ml NaCl solution (0.17 M, unbuffered) or (C) 3.00 ml suspension buffer (0.15 M NaCl, 0.02 M Tris, pH 7.4). The final concentration of BPO was held constant at 33 μ M.

Effect of Sodium Benzoate and Benzoic Acid Anhydride on Mitochondrial Respiration and Morphology of

Mitochondria. Benzoate anion is formed when mitochondria are treated with BPO; therefore, the effect of sodium benzoate on mitochondrial respiration has been tested to determine if it is involved in the mechanism by which BPO alters mitochondrial function. At concentrations that produce >90% inhibition of state 3 respiration by BPO, sodium benzoate is non-inhibitory. At concentrations that result in a maximal rate of mitochondrial swelling by BPO, sodium benzoate has no effect. Thus, the effects of BPO on mitochondrial function described herein are not caused by a decomposition product of BPO.

Benzoic acid anhydride is structurally similar to BPO except that the carbonyl groups are bridged by a single oxygen atom rather than by dioxygen. Inhibition of mitochondrial respiration and induction of mitochondrial swelling are observed with benzoic acid anhydride; however, ten times the concentration of the anhydride is required to produce approximately one half the effect seen with BPO. These results suggest that the dioxo bridge of BPO is important in the mechanism by which BPO affects mitochondrial function.

Summary. Our results show that BPO inhibits rat liver mitochondrial electron transport at coupling site II and interacts with a component of the energy-producing

assemblies. Inhibition of respiration leads to radical production in respiring SMP, although these radicals are apparently scavenged in intact respiring mitochondria. BPO also induces dose-dependent, rapid, large-amplitude swelling of mitochondria that is independent of the presence of calcium, oxygen and respiratory substrate. This swelling does not appear to be a radical process but instead a reaction of BPO at a specific locus resulting in passive osmotic swelling that is dependent on the presence of monovalent cations.

The effects of BPO on mitochondria described herein are reminiscent of the effects of BPO on keratinocytes (19), in which damage occurs via a non-radical mechanism. Whether or not the impairment of mitochondrial function is an important event in carcinogenesis remains to be established. However, the fact that organic peroxide tumor promoters (BPO, TBHP, cumene hydroperoxide) all produce alterations in the ultrastructure of the mitochondrion suggests that impairment of mitochondrial function may be a common theme in peroxide-mediated tumor promotion, albeit by different mechanisms.

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CHAPTER THREE

HYDROPEROXIDE-INDUCED RADICAL PRODUCTION IN LIVER MITOCHONDRIA

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ABSTRACT

When isolated rat liver mitochondria are treated with tert-butyl hydroperoxide in the presence of the spin trap 5,5-dimethyl-1-pyrroline-N-oxide, a six-line ESR signal is observed with parameters characteristic of a carbon-centered radical. The radical is shown to be $\text{CH}_3\cdot$ using 2-methyl-2-nitrosopropane as the spin trap. Inhibition of radical production by EDTA and N-ethylmaleimide provides evidence for participation by metals and reduced sulfhydryl groups in the radical-generating reaction. It is proposed that radicals are formed through the reaction between a reducing agent, a metal and the hydroperoxide.

ABBREVIATIONS

ESR, electron-spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; EGTA, [ethylenebis-(oxyethylenenitrilo)] tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, MNP, 2-methyl-2-nitrosopropane; NEM, N-ethylmaleimide; TBHP, tert-butyl hydroperoxide.

INTRODUCTION

The role of free radicals in the hydroperoxide-induced damage of rat liver mitochondria has been controversial. Some groups attribute the swelling observed when mitochondria are treated with hydroperoxides to lipid peroxidation initiated by radicals arising from the decomposition of the hydroperoxide (1); the initial event in this process may be a reaction between the hydroperoxide and cytochrome P-450 (2). Other groups have suggested that the swelling and loss of membrane potential in Ca^{2+} -loaded rat liver mitochondria is due to induction of Ca^{2+} cycling (3), which may be a non-radical process dependent upon pyridine nucleotide oxidation (4,5). It is surprising, therefore, that no attempt has been made to directly detect the formation of radicals from the interaction of hydroperoxides with mitochondria.

We here report a study of the action of tert-butyl hydroperoxide (TBHP) on rat liver mitochondria using the ESR spin trapping method to determine whether radicals are produced in this interaction. Spin trapping has been used to study the production of hydroxyl radicals in respiring rat heart mitochondria (6). With both Ca^{2+} -deficient, non-respiring mitochondria and Ca^{2+} -loaded, respiring mitochondria, we find that methyl radicals are produced upon incubation with TBHP. It is proposed that radicals

are generated by a metal-mediated reaction between an endogenous reducing agent (either GSH or ascorbate) and TBHP.

MATERIALS AND METHODS

Chemicals. TBHP (70%), and 2-methyl-2-nitrosopropane (MNP) were used as obtained from Aldrich. 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) was obtained from Sigma and purified by diluting with distilled, deionized water, stirring with decoloring carbon for 5 minutes and then filtering. All other chemicals were commercial samples of high purity and were used as supplied.

Preparation of Rat Liver Mitochondria. Adult male Sprague-Dawley rats (290-350 g) were fasted overnight and sacrificed by decapitation. Liver mitochondria were isolated by differential centrifugation (7) using 210 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EGTA, and 0.15% bovine serum albumin (BSA). Mitochondria were washed in the same medium without EGTA and BSA.

Determination of Protein Concentration. The protein concentration of the final mitochondrial suspension was determined by the method of Lowry, et al. (8) using BSA as a standard.

ESR Spin Trapping in Mitochondria. Mitochondria (7.7-10.7 mg mitochondrial protein) were incubated at room temperature for one minute with either DMPO (48 mM, final concentration) or MNP (55mM, final concentration) in HEPES buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.4) prior to the addition of TBHP (final concentrations shown in figure legends). After a one minute incubation in the

presence of the oxidant, the suspension was put in a 17 mm flat cell and degassed by vacuum. The flat cell was then mounted in the TM₁₁₀ wide-bore cavity for ESR measurements at X-band frequency. In some experiments, the mitochondria were solubilized in 20-100 uL of Triton X-100 before the DMPO was added. In experiments with Ca²⁺-loaded, respiring mitochondria, the incubation medium contained 24 mM succinate, 25 uM rotenone, and 1.3 mM CaCl₂. When additives (i.e., NEM and EDTA) were used, these compounds were added to the reaction mixture prior to the addition of the oxidant (final concentrations shown in Table 1). Anaerobic and aerobic incubations were performed by stirring under either nitrogen or air for 10 minutes prior to degassing and ESR measurement.

RESULTS AND DISCUSSION

Identification of Radicals Produced by TBHP in Mitochondria. When non-respiring, Ca^{2+} -deficient rat liver mitochondria are treated with TBHP in the presence of the spin trap DMPO, an ESR spectrum resulting from the trapping of two different radical intermediates is observed (Fig. 1A). The major, six-line spin adduct (Fig. 1A, closed circles) has hyperfine splitting constants (hfsc) of $a_N = 1.63$ mT and $a_H = 2.34$ mT, consistent with the adduct being a carbon-centered radical (9). The four-line signal (Fig. 1A, open circles) has hfsc of $a_N = 1.52$ mT and $a_H = 1.64$ mT, consistent with a thiyl radical spin adduct (10). There has been a report (11) that the tert-butoxyl spin adduct has similar hfsc; however, when tert-butoxyl radicals are generated by either photochemical or iron(II)-induced decomposition of tert-butyl hydroperoxide in aqueous buffer with 50 mM DMPO, we observe only the six-line signal attributed to the carbon-centered radical adduct, presumably due to methyl radicals formed by the rapid decomposition of tert-butoxyl radicals. In fact, tert-butoxyl radicals decompose to form methyl radicals and acetone much faster in water than in organic solvents (12). All of these data taken together clearly suggest that the carbon-centered spin adduct observed in our mitochondrial system is the methyl radical. The same two signals shown in Fig. 1A also are observed after the mitochondria are

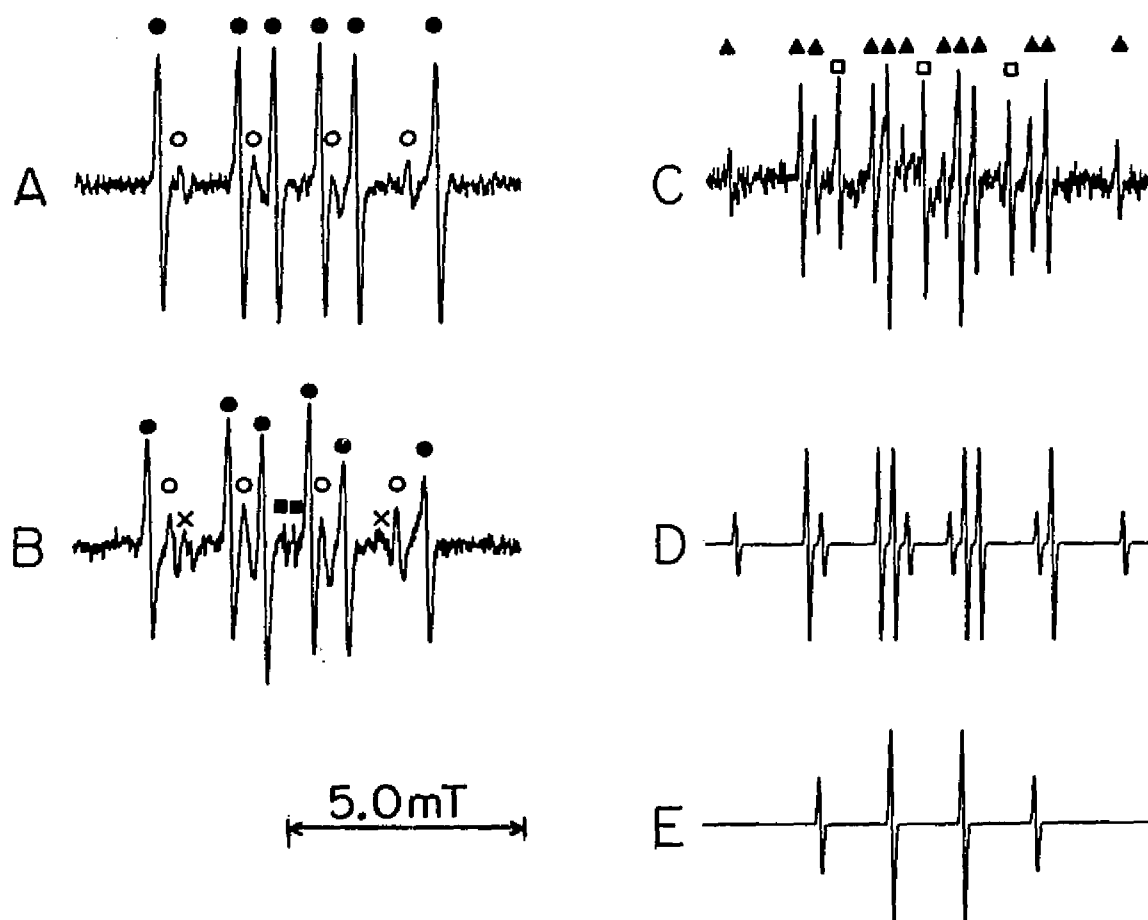


Figure 1: The ESR spectrum of radicals generated by the addition of *tert*-butyl hydroperoxide (TBHP) to rat liver mitochondria in the presence of two different spin traps. (A) Incubation containing 8.4 mg mitochondrial protein, 1.4 mM TBHP, and 49 mM DMPO in HEPES buffer, pH 7.4. (B) Same as in A except 20 μ l Triton X-100 was used to solubilize the mitochondria. (C) Same as in A except that 55 mM MNP was used as the trap. (D) Gaussian simulation of the methyl-MNP adduct using experimentally measured hfscs. (E) Gaussian simulation of the *tert*-butyl nitroxide signal using experimentally measured hfscs. All ESR measurements were carried out on an IBM Instruments model ER200-SRC spectrophotometer equipped with an Aspect 2000 data system. The following instrument conditions were used: microwave power, 20 mW; modulation amplitude, 0.32×10^{-4} T; receiver gain, 4×10^5 ; time constant, 100 ms; sweep width, 90×10^{-4} T; sweep time, 100 s; number of scans, 10.

solubilized in Triton X-100 (Fig 1B), so an intact mitochondrial membrane is not required. Weak signals due to the DMPO-superoxide spin adduct (Fig. 1B, crosses) and the ascorbyl radical (Fig. 1B, closed squares) also are observed with Triton X-100.

To further probe the identity of the carbon-centered radical, the spin trapping experiment was repeated in the presence of the spin trap MNP, and a twelve-line signal ($a_N = 1.73$ mT and $a_H = 1.43$ mT) characteristic of the MNP-methyl radical adduct was observed (Fig. 1C, closed triangles) (9). Three of the lines in the spectrum (Fig. 1C, open squares) are due to di-tert-butyl nitroxide, the photolytic decomposition product of MNP. A Gaussian simulation of the MNP-methyl radical adduct (Fig. 1D) is not completely consistent with the ratio of the peak intensities in the experimental spectrum, suggesting the presence of an overlapping four-line signal with hfsc of $a_N = 1.44$ mT and $a_H = 1.44$ mT; the Gaussian simulation of this adduct is shown in Fig. 1E. On the basis of the hfsc, we identify this signal as hydro-tert-butyl nitroxide (13). This species probably results from a one-electron reduction of MNP, followed by protonation, indicating the presence of a one-electron reducing agent.

The spin trapping experiments were also carried out with TBHP added to both respiring, Ca^{2+} -deficient mitochondria and respiring, Ca^{2+} -loaded mitochondria.

Neither respiration nor calcium loading causes significant changes in the signal observed with non-respiring, Ca^{2+} -deficient mitochondria (results not shown), implying that radicals arise from a reaction pathway that is independent of the mechanism(s) by which TBHP induces Ca^{2+} release from Ca^{2+} -loaded mitochondria (4,5).

Effect of Additives on TBHP-Induced Radical Production in Non-Respiring Mitochondria. The effect of a number of additives was investigated to elucidate the mechanism of the radical-generating reaction. The presence of a thiyl radical signal implies that a sulfur-containing compound is involved in the radical-generating reaction. N-Ethylmaleimide (NEM), which reacts with reduced sulfhydryl groups (14,15), causes a significant reduction in the intensity of the DMPO-methyl radical adduct signal (Table I) and induces the formation of an ascorbyl radical signal (Fig. 2). Interestingly, an ascorbyl signal was also observed when linoleic acid hydroperoxide was added to rat liver homogenate (16).

Many hydroperoxide reactions are metal-mediated (17,18). Consistent with this observation, EDTA partially diminishes the intensity of the DMPO-methyl radical adduct signal (Table I).

The role of oxygen in the reaction between TBHP and mitochondria was examined by incubating the reaction mixture both aerobically and anaerobically in the presence

Table I: Effect of additives on TBHP-induced radical production in mitochondria - relative ESR signal intensities

Additive ^a	Relative Signal Intensity ^b
4.9 mM N-ethylmaleimide	0.4
2.1 mM EDTA	0.3
none (aerobic incubation)	0.4
none (anaerobic incubation)	0.8

a) All reaction mixtures contained 11 mg mitochondrial protein, 1.4 mM TBHP, and 48 mM DMPO in HEPES buffer, pH 7.4.

b) The signal observed with TBHP alone (no additives, no incubation) was used as a reference. The peak of the first line of the methyl-DMPO adduct signal was used for peak height measurements.

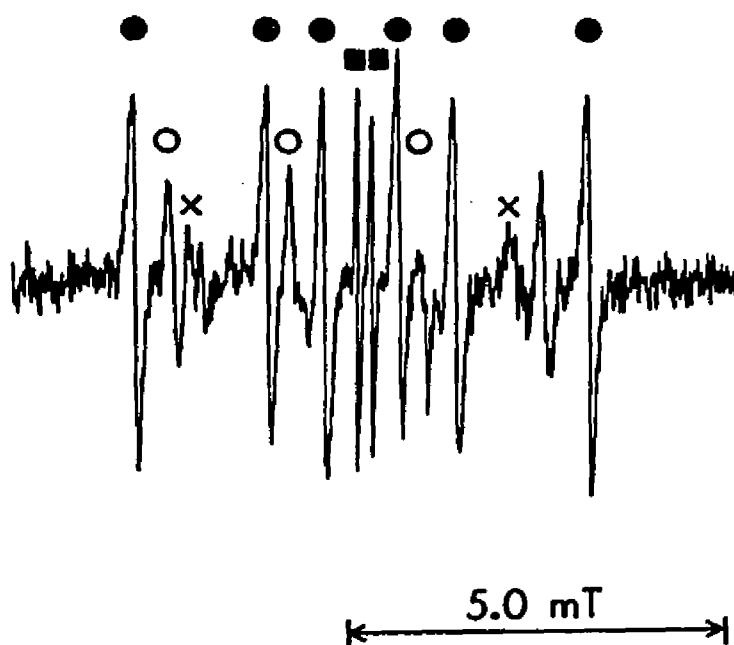


Figure 2: ESR spectrum showing the effect of sulfhydryl group depletion on the reaction between tert-butyl hydroperoxide (TBHP) and rat liver mitochondria. The reaction mixture contained 11 mg mitochondrial protein, 4.9 mM N-ethylmaleimide, 1.4 mM TBHP, and 48 mM DMPO in HEPES buffer, pH 7.4.

of DMPO. After aerobic incubation, the DMPO-methyl radical adduct signal was significantly diminished (Table I), indicating that oxygen inhibits radical production. Anaerobic incubation did not have a significant effect on radical production (Table I), although this lack of effect may be due to an inability to completely remove O₂ from the mitochondria.

These data suggest that TBHP induces radical production in mitochondria through a metal-mediated reaction between an endogenous reductant (either ascorbate or glutathione) and the hydroperoxide. These radicals could initiate lipid peroxidation in mitochondrial membranes. We are currently investigating the fate and reactions of radicals from hydroperoxide decomposition, both in mitochondria and chemical model systems and are probing the role of metal-containing enzymes (such as P450) in the reaction.

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CHAPTER FOUR

HYDROPEROXIDE-INDUCED RADICAL PRODUCTION IN LIVER MITOCHONDRIA

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Running Title: Radical Production in Mitochondria

Key Words: Electron Spin Resonance, Electron Transfer, Hydroperoxide, Lipid Peroxidation, Mitochondria, Swelling

ABSTRACT

When isolated rat liver mitochondria are treated with tert-butyl hydroperoxide (TBHP) in the presence of the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), ESR signals attributable to spin adducts are observed resulting from the trapping of methyl, tert-butyoxyl and tert-butylperoxyl radicals. The addition of respiratory substrate results in a 3- to 7.5-fold increase in the signal intensity of the methyl-DMPO adduct, no change in the signal intensity of the tert-butoxyl-DMPO adduct and complete loss of the tert-butylperoxyl-DMPO adduct. The magnitude of increase of methyl radical production in the presence of respiratory substrate is related to the respiratory control ratio (RCR) of the mitochondrial preparation. In the presence of antimycin A, which blocks electron flow between cytochromes b and c₁, no stimulation of methyl radical production is observed with respiratory substrate. This indicates that electrons are being intercepted by TBHP from a cytochrome component on the oxidase side of cytochrome b. Reduction of TBHP results in the formation of tert-butoxyl radicals, which subsequently undergo beta-scission to form methyl radicals. Stimulation of methyl radical production by the addition of respiratory substrate is observed in cytochrome c-depleted mitochondria, indicating that TBHP is reduced by cytochrome c₁. Calcium-loading of the mitochondria has no significant

effect on hydroperoxide-induced radical production. The addition of metal chelators has no significant effect on TBHP-induced radical production and radical production is inhibited by sodium cyanide in non-respiring mitochondria, suggesting that TBHP interacts with a tightly-ligated metal. Radical production is also observed when either cumene hydroperoxide or linoleic acid hydroperoxide is added to the mitochondria in the presence of spin trap.

ABBREVIATIONS

BHB, beta-hydroxy butyrate; BSA, bovine serum albumin; CCCP, carbonyl cyanide m-chlorophenylhydrazine; CHP, cumene hydroperoxide; ESR, electron-spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DMPOX, 5,5-dimethyl-1-pyrrolidone-2-oxyl radical; EDTA, ethylenediamine tetraacetic acid; EGTA, [ethylenedis-(oxyethylenenitrilo)] tetraacetic acid; GSH, reduced glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LHP, linoleic acid hydroperoxide; MNP, 2-methyl-2-nitrosopropane; MSA, mercaptosuccinic acid; NEM, N-ethylmaleimide; PBN, N-tert-butyl-alpha-phenylnitrone; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid; TBHP, tert-butyl hydroperoxide; TCA, trichloroacetic acid.

INTRODUCTION

Skin carcinogenesis can be divided into initiation, promotion and progression stages. Tumor initiation is an irreversible step that arises from the interaction of a carcinogen with DNA resulting in gene alteration (1). During the promotion stage, the altered genetic material of the initiated cell becomes expressed through selection and clonal expansion (2). The result of initiation followed by promotion is commonly the formation of a high yield of benign tumors. The progression of tumors from a benign to malignant neoplasm occurs in the final stage of carcinogenesis; this transformation is proposed to involve further genetic changes caused by mutagenic agents (3,4). This model of carcinogenesis has been shown to exist in a number of systems including the liver (5).

Several peroxidic compounds are active as tumor promoters; benzoyl peroxide (BPO), cumene hydroperoxide (CHP), decanoyl peroxide, dicumyl peroxide, and lauroyl peroxide are strong promoters while tert-butyl hydroperoxide (TBHP), hydrogen peroxide, and methyl ethyl ketone peroxide are less active (6-10). Although free radicals have been implicated in tumor promotion (11,12), there is little direct evidence that the tumor promoting activity of peroxidic compounds is due to their ability to induce radical production.

A number of tumor promoters have been shown to induce epidermal hyperplasia and the formation of dark basal keratinocytes in skin; the number of these induced cells correlates well with the tumor-promoting activity of several promoters (13,14). Although TBHP and cumene hydroperoxide have been shown by ESR spin-trapping studies to induce radical production in keratinocytes (15), the significance of these radicals in the process of tumor promotion is unknown. The radical process of lipid peroxidation has been shown to be inducible in keratinocytes but is unrelated to cell damage (16); BPO is cytotoxic to these cells but does not induce lipid peroxidation (16). The cytotoxicity of BPO may be explained by the fact that it inhibits mitochondrial electron- and energy-transfer and induces rapid, large-amplitude swelling of mitochondria (17). Thus, the mitochondrion may be an important organelle in the interaction between peroxide tumor promoters and whole cells.

In cancer cells, mitochondrial function is reported to be impaired (18); these impairments include changes in ultrastructure, defective energy-linked functions, and altered Ca^{2+} transport. Prooxidant promoters, including organic peroxides, have been shown to cause similar changes in mitochondrial function. Although peroxidic tumor promoters are known to impair normal mitochondrial

function, it remains to be established whether such impairment is involved in the tumor promotion process.

Peroxide-induced mitochondrial damage occurs by both radical and non-radical pathways. CHP has been shown to yield radicals that initiate mitochondrial lipid peroxidation by a mechanism that involves mitochondrial cytochrome P-450 (19). In contrast, BPO alters mitochondrial function by an apparently non-radical process (17). The interaction of TBHP with mitochondria has been shown to occur by both radical and non-radical pathways; for example, TBHP initiates mitochondrial swelling via a non-radical process that is dependent on Ca^{2+} cycling (20-22). However, we previously reported that TBHP forms free radicals in mitochondria as shown by ESR spin trapping (23).

Herein, we report a study of the mechanism of hydroperoxide-induced radical production in rat liver mitochondria using the ESR spin trapping method.

MATERIALS AND METHODS

Chemicals. tert-Butyl hydroperoxide (TBHP), Cumene hydroperoxide (CHP), linoleic acid and 2-methyl-2-nitrosopropane (MNP) were obtained from Aldrich. N-tert-butyl-alpha-phenylnitron (PBN) was from Kodak. Bovine serum albumin (BSA), catalase, glutathione peroxidase, soybean lipoxygenase (type 1-S) and superoxide dismutase were purchased from Sigma. Desferal (desferrioxamine mesylate) was provided by Ciba-Geigy. 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) was obtained from Sigma and purified by diluting with distilled, deionized water, stirring with decoloring carbon for 5 minutes and then filtering. Linoleic acid hydroperoxide (LHP) was prepared and isolated according to the method of Gardner (24). All other chemicals were commercial samples of high purity and were used as supplied.

Preparation of Rat Liver Mitochondria. Adult male Sprague-Dawley rats (175-275 g) were fasted overnight and sacrificed by decapitation. Liver mitochondria were isolated by differential centrifugation (25) using 210 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, and 0.15% bovine serum albumin (BSA). Mitochondria were washed in the same medium without EDTA, EGTA, and BSA. Mitochondria prepared for the TBA test were washed in 150 mM NaCl. Cytochrome c-depleted mitochondria were prepared by treatment with hypotonic medium followed

by washing with salt solution according to the method of Jacobs and Sanadi (26).

Determination of Protein Concentration. The protein concentration of the mitochondrial suspensions were determined by the method of Bradford (27) using BSA as a standard.

ESR Spin Trapping in Mitochondria. Rat liver mitochondria (5.0 mg mitochondrial protein) were incubated at room temperature for one minute with either DMPO (50 mM, final concentration), MNP (55 mM, final concentration) or PBN (50 mM, final concentration) in MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.4) prior to the addition of the hydroperoxide (final concentrations shown in figure legends). After a 1 min incubation in the presence of the oxidant, the suspension was placed in a 17 mm flat cell and degassed by vacuum. The flat cell was then mounted in the TM₁₁₀ wide-bore cavity for ESR measurements at X-band frequency. All ESR measurements were initiated 3 min after the addition of the oxidant. In experiments with respiring mitochondria, either sodium succinate (10 mM, final concentration) or beta-hydroxybutyrate (10 mM, final concentration) was added to the mitochondria 1 min prior to the addition of hydroperoxide. In some experiments, the mitochondria were solubilized in Triton X-100 (1.3% v/v, final concentration) prior to the addition of DMPO. When additives were used,

these compounds were added to the reaction mixture prior to the addition of the oxidant (final concentrations shown in Tables I and II). Anaerobic and aerobic incubations were performed by purging the mitochondria with either nitrogen or oxygen for 5 min prior to the addition of TBHP.

Measurement of Lipid Peroxidation by the TBA Test.

Peroxidation of mitochondrial membrane lipids was assessed by the spectrophotometric determination of TBA-reactive products according to the procedure described by Aust (28). Coupled rat liver mitochondria (6.0 mg protein) were added to 6.0 ml (final volume) of medium saturated with air at 25°C and containing 150 mM NaCl at a final pH of 7.4. A 1.0 ml aliquot was removed and added to 2.0 ml of stock TBA-TCA-HCl reagent (15% w/v TCA, 0.375% TBA, 0.25 N HCl). Either mercaptosuccinic acid (MSA), sodium succinate, TBHP or a combination of these compounds were added to the remaining mitochondria (final concentrations given in Figure 8). Aliquots (1.0 ml) were removed at 30, 60, 90, and 120 min following the addition of the initiator and added to stock TBA-TCA-HCl reagent. The samples were placed in a boiling water bath for 15 min, cooled and centrifuged to remove the precipitate formed during boiling. The absorbance of the samples was measured at 532 nm on a Hewlett Packard UV-visible spectrophotometer against a blank that contained all the reagents but no mitochondria.

RESULTS AND DISCUSSION

Identification of Radicals Produced by Hydroperoxides in Mitochondria. When non-respiring, Ca^{2+} -deficient rat liver mitochondria are treated with TBHP in the presence of the spin trap DMPO, an ESR spectrum resulting from the trapping of three different radical intermediates is observed (Fig. 1A). Gaussian simulations of the individual adducts are shown in Fig. 1, C-E. The hyperfine splitting constants (hfsc) of the major, six-line signal ($a_N = 1.66$ mT, $a_H = 2.38$ mT) (Fig. 1C) identify it as a carbon-centered radical adduct (29). The four-line signal ($a_N = 1.49$ mT, $a_H = 1.67$ mT) (Fig. 1D) could be either a thiyl radical spin adduct (30) or a tert-butoxyl spin adduct (31). The twelve-line signal ($a_N = 1.48$ mT, $a_H = 1.09$ mT, $a_H = 0.15$ mT) (Fig. 1E) is identified as a peroxy spin adduct (32), although the possibility of trapping superoxide to form a hydroperoxyl adduct must be considered. A simulation of the combination of these adducts (Fig. 1B) compares well to the experimental spectrum (Fig. 1A). When mitochondria are heat-denatured prior to the addition of TBHP, a significant reduction in radical production is observed (Fig. 1F), indicating that TBHP reacts with a heat-labile component of mitochondria. No radical adducts are observed in the absence of the hydroperoxide (Fig. 1G).

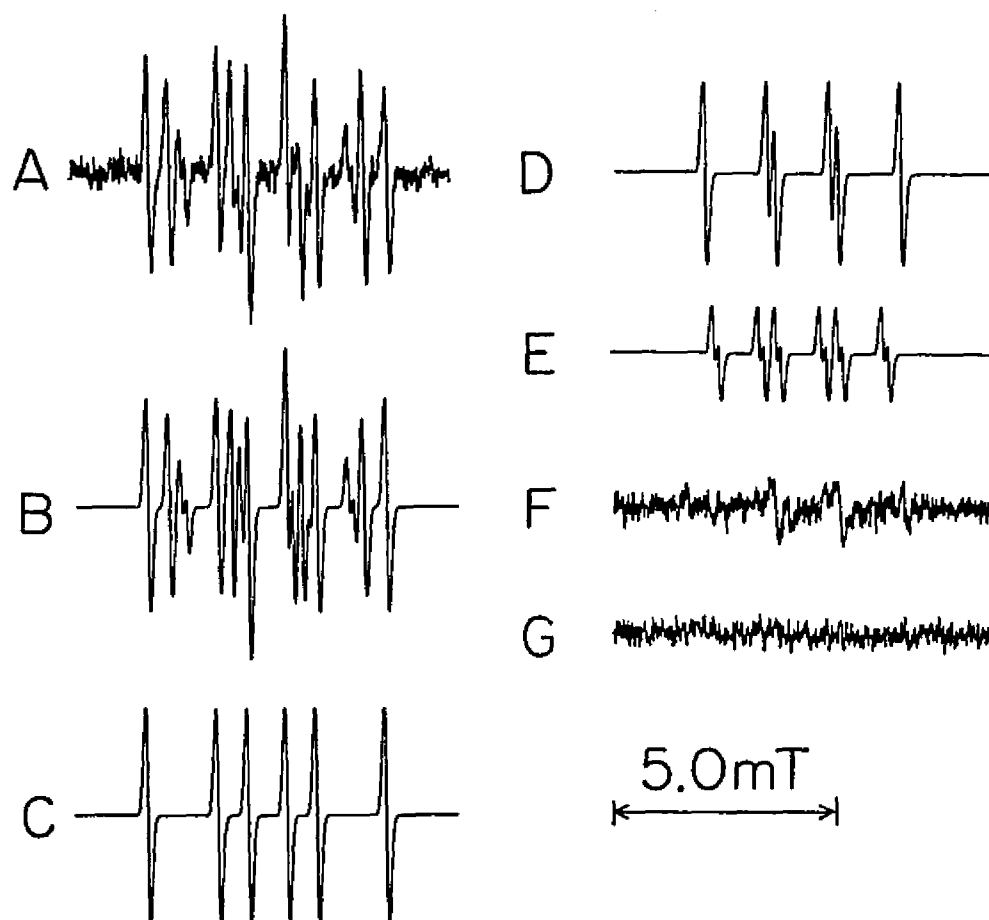


Figure 1: The ESR spectrum of radicals generated by the addition of tert-butyl hydroperoxide (TBHP) to rat liver mitochondria in the presence of DMPO. (A) Incubation containing 5.0 mg mitochondrial protein, 10 mM TBHP, and 50 mM DMPO in MSH buffer, pH 7.4. (B) Gaussian simulation of spectra C-E. (C) Gaussian simulation of DMPO-carbon-centered adduct. (D) Gaussian simulation of DMPO-tert-butoxyl adduct. (E) Gaussian simulation of DMPO-tert-butylperoxyl adduct. (F) Same as in A except mitochondria were heat-denatured prior to addition of DMPO and TBHP. (G) Same as in A except no TBHP was added. ESR measurements were carried out on an IBM Instruments model ER100D spectrophotometer equipped with an Aspect 2000 data system. The following instrument conditions were used: microwave power, 20 mW; modulation amplitude, 0.32×10^{-4} T; receiver gain, 4×10^5 ; time constant, 100 ms; sweep width, 90×10^{-4} T; sweep time, 100 s; number of scans, 10.

The identity of the carbon-centered radical has been probed by repeating the spin trapping experiment in the presence of the spin trap MNP. A spectrum consisting of three spin adducts is observed (Fig. 2A). Gaussian simulations of the individual adducts are shown in Fig. 2, C-E. The twelve-line signal (Fig. 2C) has hfsc characteristic of the MNP-methyl radical adduct ($a_N = 1.73$ mT, $a_H = 1.43$ mT) (29). The simulation of the MNP-methyl radical adduct (Fig. 2C) is not completely consistent with the ratio of the peak intensities in the experimental spectrum, suggesting the presence of an overlapping four-line signal ($a_N = 1.44$ mT, $a_H = 1.44$ mT) (Fig. 2D). On the basis of these hfsc, we identify this signal as due to hydro-tert-butyl nitroxide (33). Three of the lines in the spectrum are due to di-tert-butyl nitroxide ($a_N = 17.1$ mT), the photolytic decomposition product of MNP (Fig. 2E) (34). When these simulations are combined, the resulting spectrum (Fig. 2B) compares well to the experimental spectrum (Fig. 2A).

The identity of the four-line DMPO spin adduct (Fig. 1A) has been probed by generating bona-fide thiyl-DMPO and tert-butoxyl-DMPO adducts. The thiyl-DMPO adduct ($a_N = 1.54$ mT, $a_H = 1.62$ mT) was generated by photolysis of 10 mM oxidized glutathione (GSSG) in the presence of 50 mM DMPO. The tert-butoxyl-DMPO adduct ($a_N = 1.50$ mT, $a_H = 1.68$ mT) was generated by reacting 0.1 mM FeSO_4 with 1.5 mM TBHP in

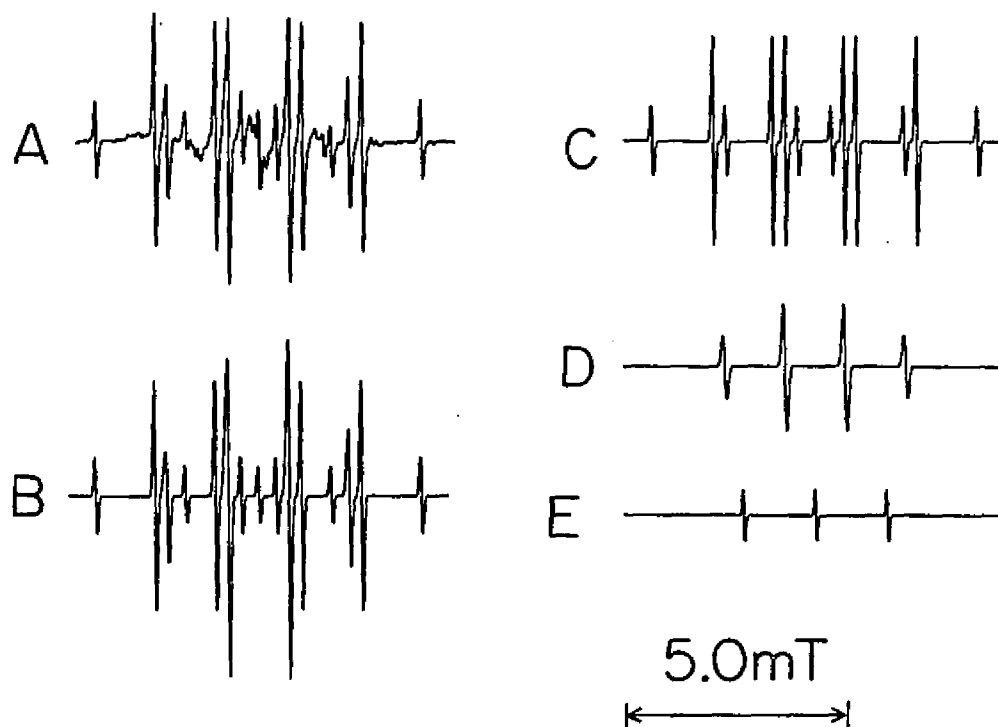


Figure 2: The ESR spectrum of radicals generated by the addition of tert-butyl hydroperoxide (TBHP) to rat liver mitochondria in the presence of MNP. (A) Incubation containing 8.4 mg mitochondrial protein, 1.4 mM TBHP, and 55 mM MNP in MSH buffer, pH 7.4. (B) Gaussian simulation of spectra C-E. (C) Gaussian simulation of the methyl-MNP adduct. (D) Gaussian simulation of the hydro-tert-butyl nitroxide signal. (E) Gaussian simulation of the di-tert-butyl nitroxide signal. Instrument conditions same as in Figure 1 except ESR measurements were carried out on an IBM Instruments model ER200-SRC spectrophotometer.

the presence of 50 mM DMPO. On the basis of these hfsc values, we identify the four-line adduct ($a_N = 1.49$ mT, $a_H = 1.67$ mT) produced when mitochondria are treated with TBHP in the presence of DMPO as the tert-butoxyl-DMPO adduct.

Superoxide dismutase (SOD) is commonly used to distinguish between trapping of superoxide (as the hydroperoxyl adduct) and peroxy radicals (35). Due to its size, SOD would not be expected to cross an intact mitochondrial membrane. To circumvent this problem, we have used detergent-solubilized mitochondria. The same signals shown in Fig. 1A also are observed after the mitochondria are solubilized in Triton X-100 and then treated with TBHP in the presence of DMPO; therefore, an intact mitochondrial membrane is not required to observe hydroperoxide-induced radical production. We have shown that SOD is active in the presence of Triton X-100 by adding it to a detergent-solubilized xanthine/xanthine oxidase system in the presence of DMPO; in the absence of SOD, a twelve-line superoxide adduct is observed while no signal is observed in the presence of SOD. When Triton X-solubilized mitochondria are treated with TBHP in the presence of SOD and DMPO, no change is observed in the intensity of the twelve-line adduct, suggesting that this adduct is the tert-butylperoxyl-DMPO adduct and not the hydroperoxyl-DMPO adduct.

When cumene hydroperoxide is added to non-respiring, Ca^{2+} -deficient rat liver mitochondria in the presence of DMPO, three radical intermediates are trapped (Fig. 3A). In this case, the twelve-line signal ($a_N = 1.47$ mT, $a_H = 1.08$ mT, $a_H = 0.15$ mT) is dominant. The intensity of the six-line signal ($a_N = 1.66$ mT, $a_H = 2.38$ mT) is significantly reduced while the four-line signal (intensity too low to measure hfscs) is almost non-existent. On the basis of the similarity of these signals to those observed in the TBHP system, we assign the twelve-line signal to a peroxy-DMPO adduct and the six-line signal to the methyl-DMPO adduct. Gaussian simulations of these adducts are shown in Fig. 3, C-E. The simulation of the combination of these adducts (Fig. 3B) compares well with the experimental spectrum (Fig. 3A).

When linoleic acid hydroperoxide (LHP) is added to mitochondria in the presence of DMPO, only a very weak 6-line ESR signal is observed (Fig. 4A). This result may be due to the inability of LHP to generate radicals in this mitochondrial system or to the fact that DMPO is a hydrophilic spin trap. In the latter case, radicals generated by the decomposition of LOOH in a lipophilic environment may not be trapped by DMPO. To probe this possibility, mitochondria were treated with LOOH in the presence of PBN, a lipophilic spin trap; a spectrum resulting from the trapping of a carbon-centered radical

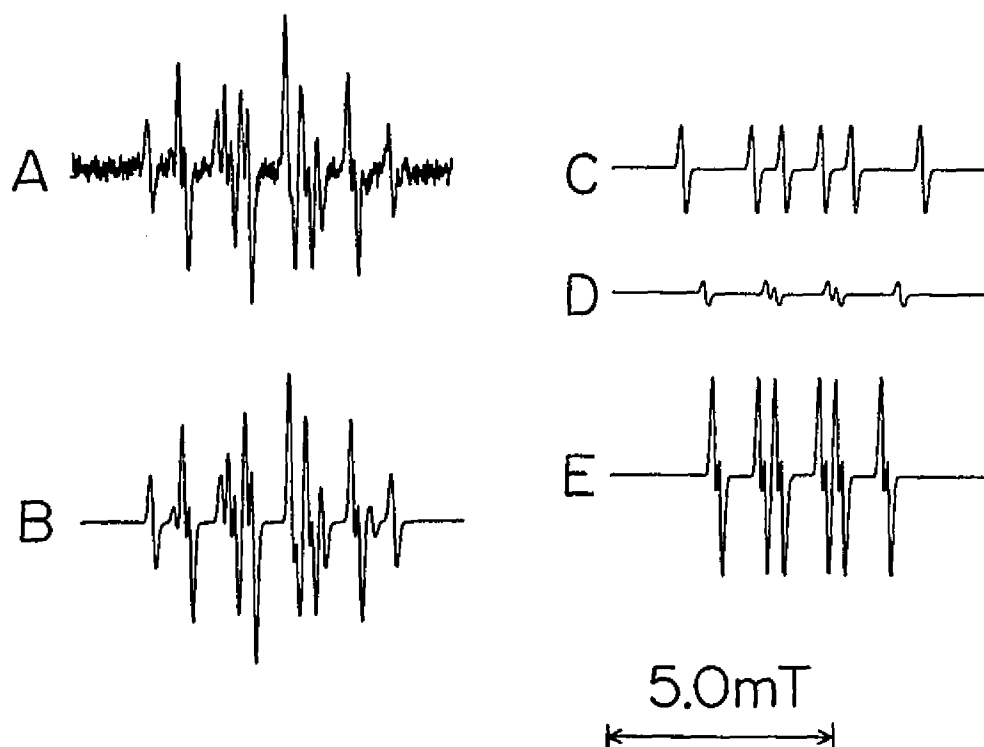


Figure 3: The ESR spectrum of radicals generated by the addition of cumene hydroperoxide (CHP) to rat liver mitochondria in the presence of DMPO. (A) Incubation containing 5.0 mg mitochondrial protein, 10 mM CHP, and 50 mM DMPO in MSH buffer, pH 7.4. (B) Gaussian simulation of spectra C-E. (C) Gaussian simulation of DMPO-carbon-centered adduct. (D) Gaussian simulation of DMPO-cumyloxyl adduct. (E) Gaussian simulation of DMPO-cumylperoxyl adduct. Instrument conditions same as in Figure 1.

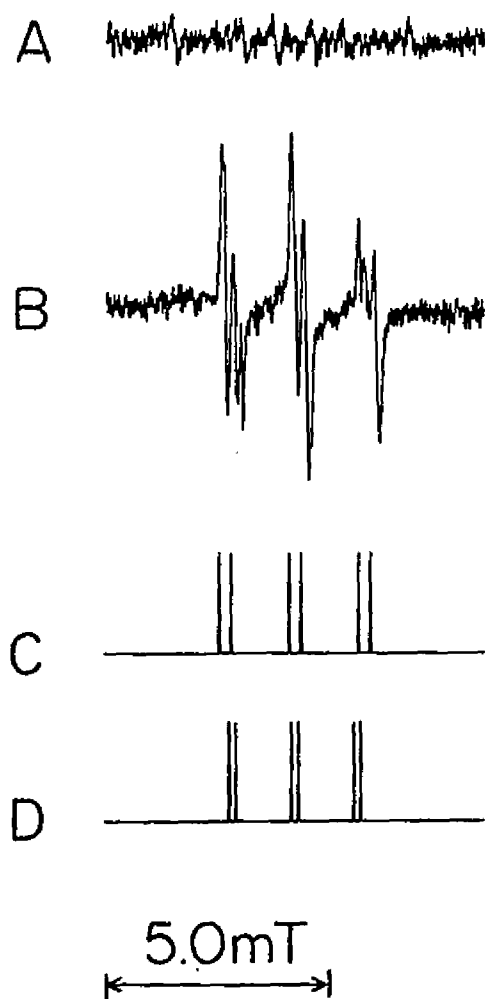


Figure 4: The ESR spectrum of radicals generated by the addition of linoleic acid hydroperoxide (LHP) to rat liver mitochondria in the presence of either DMPO or PBN. (A) Incubation containing 5.0 mg mitochondrial protein, 10 mM LHP, and 50 mM DMPO in MSH buffer, pH 7.4. (B) Same as in A except 50 mM PBN was used instead of DMPO. (C) Stick simulation of carbon-centered-PBN adduct in A. (D) Stick simulation of oxygen-centered-PBN adduct in A. Instrument conditions same as in Figure 1.

($a_N = 1.63$ mT, $a_H = 0.28$ mT) and an oxygen-centered radical ($a_N = 1.46$ mT, $a_H = 0.16$ mT) is observed (Fig. 4B). Stick simulations of these adducts are shown in Fig. 4C and 4D. Our measured hfsc values for these adducts are consistent with literature values for PBN adducts of L-C \cdot (36) and LO \cdot (37) respectively. These results indicate that LHP does induce radical production in mitochondria and suggest that generation of radicals occurs in a non-aqueous environment. No increase in DMPO spin adduct formation is observed when Triton X-solubilized mitochondria are treated with LHP compared to that seen in unsolubilized mitochondria (Fig. 4A). Thus, the inability to trap radicals in LHP-induced radicals in mitochondria with DMPO is a function of the spin trap (i.e., rate of trapping and/or stability of adducts) and not the environment of radical production.

PBN spin adducts are also observed when mitochondria are treated with either TBHP (Fig. 5A) or CHP (Fig. 5D) in the presence of this spin trap. In each case, a spectrum resulting from the trapping of two radical species is observed. TBHP (Fig. 5A) induces the production of both a carbon-centered radical ($a_N = 1.67$ mT, $a_H = 0.35$ mT) and an oxygen-centered radical ($a_N = 1.53$ mT, $a_H = 0.35$ mT). Stick simulations of the individual adducts are shown in Fig. 5B and 5C. These hfsc values are consistent with literature values (38) for the PBN-methyl radical adduct ($a_N = 1.66$ mT, $a_H = 0.36$ mT) and PBN-tert-butoxyl radical

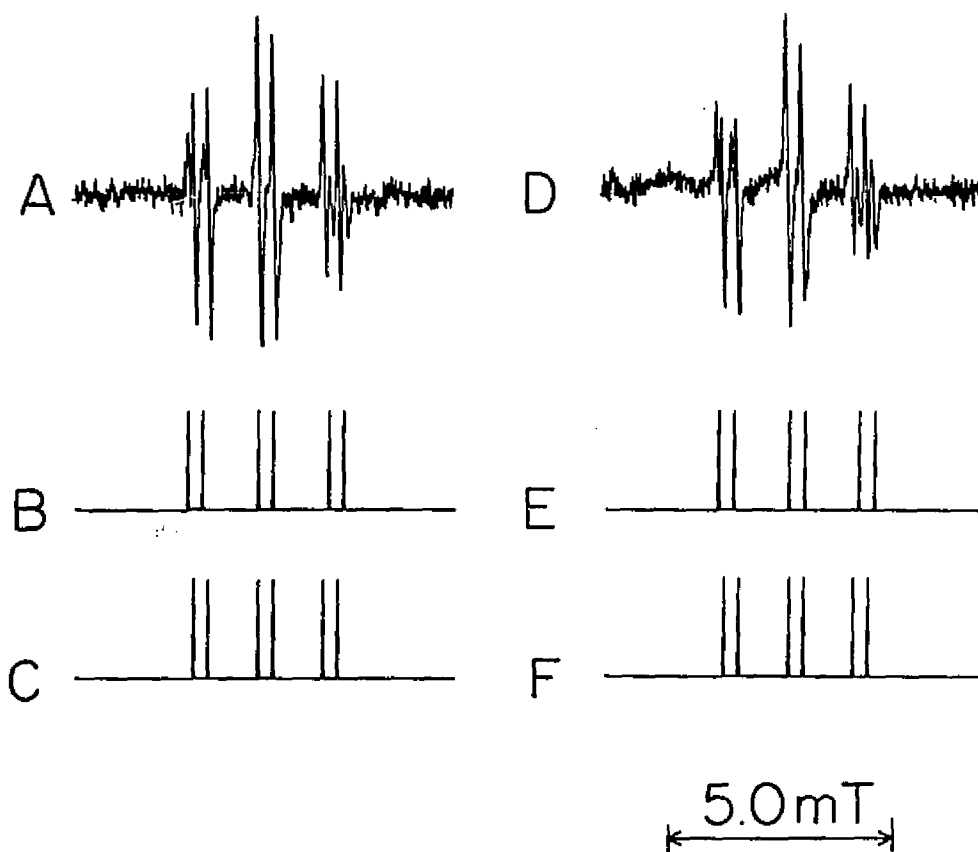


Figure 5: The ESR spectrum of radicals generated by the addition of either tert-butyl hydroperoxide (TBHP) or cumene hydroperoxide (CHP) to rat liver mitochondria in the presence of PBN. (A) Incubation containing 5.0 mg mitochondrial protein, 10 mM TBHP, and 50 mM PBN in MSH buffer, pH 7.4. (B) Stick simulation of carbon-centered-PBN adduct in A. (C) Stick simulation of oxygen-centered-PBN adduct in A. (D) Same as in A but 10 mM CHP added instead of TBHP. (E) Stick simulation of carbon-centered-PBN adduct in D. (F) Stick simulation of oxygen-centered-PBN adduct in D. Instrument conditions same as in Figure 1.

adduct ($a_N = 1.53$ mT, $a_H = 0.33$ mT). CHP (Fig. 5D) also induces the production of both a carbon-centered radical ($a_N = 1.67$ mT, $a_H = 0.37$ mT) and an oxygen-centered radical ($a_N = 1.53$ mT, $a_H = 0.35$ mT). Stick simulations of the individual adducts are shown in Fig. 5E and 5F. The carbon-centered adduct can likewise be identified as the PBN-methyl radical adduct. The oxygen-centered adduct may be due to trapping of either cumyloxyl or cumylperoxyl radicals. Since the peroxyl-DMPO adduct is the major signal when CHP is added to mitochondria in the presence of DMPO (Fig. 3A), the oxygen-centered adduct in Fig. 5D is probably also a peroxyl adduct, although it is difficult to distinguish between alkoxyl and peroxyl adducts with PBN.

Effect of Additives on TBHP-Induced Radical Production in Respiring Mitochondria. When sodium succinate, a $FADH_2$ -linked substrate, is added to mitochondria prior to the addition of TBHP, a significant enhancement in the intensity of the methyl-DMPO adduct is observed (Fig. 6). A greater stimulation in radical production is observed as the respiratory control ratio (RCR) increases. For example, a mitochondrial preparation with a RCR of 2.7 yielded a 2.9-fold stimulation of TBHP-induced radical production in the presence of substrate whereas a mitochondrial preparation with a RCR of 5.2 resulted in a 7.6-fold stimulation. In uncoupled mitochondria, the rate of state 4 respiration is increased compared to tightly

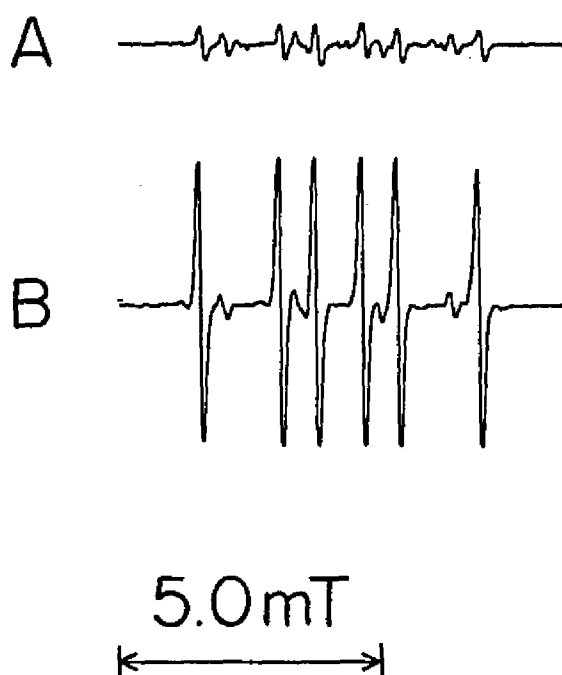


Figure 6: The effect of respiratory substrate on tert-butyl hydroperoxide (TBHP)-induced radical production in rat liver mitochondria. (A) Incubation containing 5.0 mg mitochondrial protein, 1.5 mM TBHP, and 50 mM DMPO in MSH buffer, pH 7.4. (B) same as in A but 10 mM sodium succinate added prior to TBHP. Instrument conditions same as in Figure 1.

coupled preparations. When mitochondria are treated with the uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) prior to the addition of sodium succinate and TBHP, a 40% inhibition of substrate-enhanced, TBHP-induced radical production is observed (Table I). This result suggests a lower electron acceptor efficiency of TBHP under conditions of an increased rate of electron transport.

When beta-hydroxybutyrate (BHB), a NADH-linked substrate, is added to mitochondria prior to TBHP, a 5.1-fold increase in radical production is observed when a mitochondrial preparation with an RCR of 4.1 is used. The fact that both NADH- and FADH₂-linked substrates stimulate TBHP-induced radical production in mitochondria suggests either that TBHP interacts at a site other than NADH-oxidase and succinoxidase or that TBHP interacts with both of these sites. The ability of respiratory substrate to enhance TBHP-induced radical production in mitochondria was studied in the presence of inhibitors of mitochondrial electron transport to better identify a locus of interaction of TBHP (Table I). Rotenone, a NADH-oxidase inhibitor (Fig. 7), inhibited radical production when added to mitochondria prior to BHB. The intensity of methyl-DMPO adduct measured in the presence of rotenone is equal to that seen in the absence of respiratory substrate, suggesting that TBHP does not accept electrons from NADH-

Table I: Effects of additives on TBHP-induced radical production in respiring mitochondria - relative ESR signal intensities.

Additive ^a	Relative Signal Intensity ^b
3.6 μ M CCCP	0.6
4.9 μ M rotenone ^c	0.2
0.65 μ M antimycin A	0.2
0.1 mM desferal	0.9
0.23 mM CaCl_2	1.2
None (anaerobic incubation) ^d	1.1

- a) All reaction mixtures contained 5 mg mitochondrial protein, 10 mM succinate, 4.9 μ M rotenone, 1.5 mM TBHP, and 50 mM DMPO in MSH buffer, pH 7.4 (except where noted).
- b) The intensity of the signal in the presence of the additive was divided by the intensity of the signal with respiratory substrate and TBHP alone (no additives, no incubation). The low-field peak of the methyl-DMPO adduct was used for peak height measurements. All experiments were performed in duplicate.
- c) 10 mM β -OH butyrate was used as the respiratory substrate instead of succinate. In the reference experiments using this substrate, no rotenone was added.
- d) Mitochondria were purged with nitrogen for 5 min prior to the addition of TBHP.

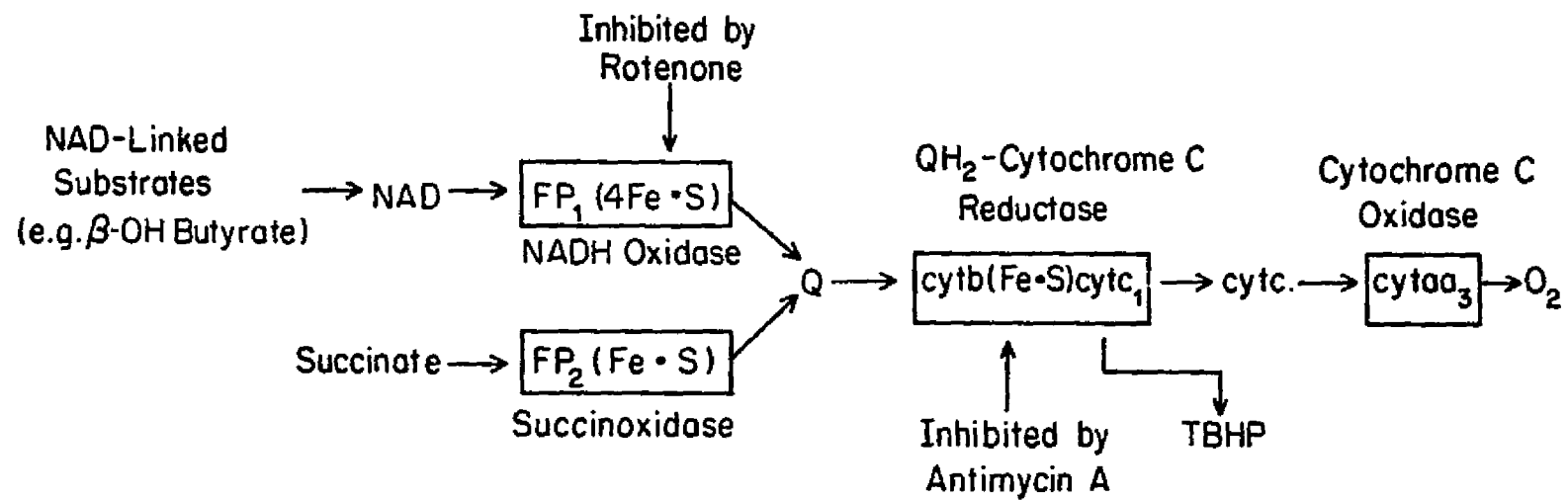


Figure 4: The mitochondrial electron transport chain.

oxidase. When antimycin A, an inhibitor of coenzyme Q-cytochrome c reductase (Fig. 7), is added to mitochondria prior to sodium succinate, inhibition of radical production is observed. This result suggests that TBHP does not accept electrons from a chain component on the substrate side of cytochrome c_1 . Thus, on the basis of these results, TBHP must interact with either cytochrome c_1 or a cytochrome on the oxidase side of this electron carrier.

The addition of desferrioxamine to mitochondria prior to respiratory substrate results in no significant inhibition of TBHP-induced radical production (Table I). Although desferrioxamine is an efficient ferric iron chelator, it does not remove iron from heme groups (32). This suggests either that the metal center involved in the reaction with TBHP is tightly bound to ligands (e.g., heme iron) or that the metal is not iron (which is a possibility since there is a copper center in cytochrome oxidase).

TBHP has been shown to interact with ferricytochrome c in submitochondrial particles (39) and in an isolated, purified state (40). It has been proposed that the hydroperoxide binds to the sixth ligand position of the iron (41,42) and is then cleaved via a cytochrome c-catalyzed homolytic rupture of the oxygen-oxygen bond of the hydroperoxide (43,44). Davies (32) has observed that interaction of TBHP with ferricytochrome c in the presence of DMPO results in the production of alkoxyl-, peroxy- and

methyl-DMPO adducts as well as 5,5-dimethyl-1-pyrrolidone-2-oxyl radical (DMPOX). To establish whether TBHP interacts specifically with either cytochrome c_1 or cytochrome c , mitochondria have been depleted of cytochrome c and then treated with sodium succinate and TBHP in the presence of DMPO. Enhancement of TBHP-induced radical production by respiratory substrate is observed in this system, indicating that at least the majority of electron interception by TBHP must take place at cytochrome c_1 .

TBHP is known to induce calcium cycling in mitochondria by a mechanism that is dependent on the metabolism of the hydroperoxide by mitochondrial glutathione peroxidase (20-23). We have examined TBHP-induced radical production in calcium-loaded, respiring mitochondria to determine whether calcium cycling is important in the mechanism of radical production. Calcium loading did not cause any significant change in radical production (Table I), implying that radicals arise from a reaction pathway that is independent of the mechanism(s) by which TBHP induces Ca^{2+} release from Ca^{2+} -loaded mitochondria (20-23).

The effect of oxygen on TBHP-induced radical production has been investigated by purging mitochondria with N_2 for 5 min prior to the addition of respiratory substrate and TBHP. Anaerobic incubation has no significant effect on methyl radical production (Table I).

Thus, oxygen does not appear to limit electron supply to TBHP.

Effect of Additives on TBHP-Induced Radical Production in Non-Respiring Mitochondria. Enhancement of methyl radical production upon the addition of either ascorbic acid or GSH to mitochondria (Table II) may be due to reduction of endogenous iron by these compounds followed by electron transfer to TBHP. It is not surprising that no alkoxyl-DMPO or peroxy-DMPO adducts are observed when ascorbic acid is present (data not shown) since this compound is a good scavenger of oxygen-centered radicals (45). It is also believed to enhance the radical-scavenging ability of vitamin E by reducing oxidized alpha-tocopherol (46). Thus, ascorbic acid may act as both a stimulator and inhibitor of radical production in the mitochondria/TBHP system.

Because many hydroperoxide reactions are metal-mediated (47,48), the effect of metal chelators on TBHP-induced radical production in mitochondria was studied. The 30% inhibition of radical production by desferrioxamine (Table II) can be rationalized by the ability of this compound to scavenge oxygen-centered radicals. The inhibition of lipid peroxidation by desferrioxamine has been attributed to the ability of this compound to act as an electron or hydrogen atom donor rather than by chelation of iron (49). EDTA only reduces TBHP-induced radical

Table II: Effects of additives on TBHP-induced radical production in non-respiring mitochondria - relative ESR signal intensities.

Additive ^a	Relative Signal Intensity ^b
0.5 mM ascorbic acid	1.5
0.5 mM GSH	1.7
0.1 mM desferal	0.7
2.0 mM EDTA	0.8
9.6 mM sodium cyanide	0.3
0.2 mM FeCl ₃	0.9
0.2 mM CuCl ₂	0.9
4.9 mM NEM	0.5
5.0 mM DTNB	1.0
none (anaerobic incubation) ^c	1.7
none (aerobic incubation) ^d	1.0
carbon monoxide ^e	2.0
1 unit/ml GSH-peroxidase + 3.3 mM GSH ^f	0.3
1.0 mg/ml BSA ^f	0.6
1.0 mg/ml catalase ^f	0.6
50 ug/ml superoxide dismutase ^f	1.1

- a) All reaction mixtures contained 5 mg mitochondrial protein, 1.5 mM TBHP, and 50 mM DMPO in MSH buffer, pH 7.4.
- b) The intensity of the signal in the presence of the additive was divided by the intensity of the signal with TBHP alone (no additives, no incubation). The low-field peak of the methyl-DMPO adduct was used for peak height measurements. All experiments were performed in duplicate.
- c) Mitochondria were purged with nitrogen for 5 min prior to the addition of TBHP.
- d) Mitochondria were purged with oxygen for 5 min prior to the addition of TBHP.
- e) Mitochondria were purged with carbon monoxide for 30 sec prior to the addition of TBHP.
- f) Mitochondria were solubilized with Triton X-100 (1.3 % v/v) prior to addition of protein and TBHP.

production by 20% (Table II). The fact that these chelators are unable to completely inhibit radical production suggests either that, as in the respiring system, the metal center which interacts with TBHP is tightly bound to ligands or that the reaction is not metal-mediated. To further probe these possibilities, sodium cyanide, a compound known to react with heme iron (50), was added to mitochondria prior to TBHP. Significant inhibition of radical production by sodium cyanide (Table II) suggests that this compound interferes with the reaction between TBHP and a metal center either by altering the redox potential of the liganded metal (51,52) such that it inhibits charge transfer between the metal and peroxide or by inhibiting the binding of TBHP to the metal center (40).

Both ferric ions and cupric ions were added to mitochondria to determine whether the interaction of TBHP and free metals results in enhanced radical production in these organelles. Neither FeCl_3 nor CuCl_2 added to mitochondria prior to TBHP significantly effected radical production (Table II) suggesting that interaction of non-liganded metals with TBHP is not an important reaction in this system. Taken as a whole, the chelator, sodium cyanide and metal (Fe and Cu) results suggest that TBHP interacts with a tightly bound metal center (e.g., heme iron) in the mitochondria.

We have investigated the effects of treating mitochondria with reagents that derivatize reduced sulfhydryl groups (53-55). The addition of N-ethylmaleimide (NEM) causes a 50% reduction in the intensity of the DMPO-methyl radical adduct signal (Table II), suggesting that sulfhydryl groups are involved in the mechanism of radical production. The addition of 5,5,-dithiobis(2-nitrobenzoic acid) (DTNB) provides conflicting results, as it has no significant effect on radical production (Table II). This lack of effect may be due to either the size or charge of this compound, both of which may prohibit it from reaching the site of NEM interaction. NEM may inhibit TBHP-induced radical production by reacting with reduced sulfhydryl groups in a heme protein resulting in a change in conformation such that the interaction between TBHP and heme iron is less efficient.

The role of oxygen in the reaction between TBHP and non-respiring mitochondria was examined by purging the mitochondria with either oxygen (aerobic incubation) or nitrogen (anaerobic incubation) prior to the addition of TBHP. Anaerobic incubation results in a 70% increase in the intensity of the methyl-DMPO adduct (Table II). Aerobic incubation has no effect on radical production (Table II). The latter result indicates that normal atmospheric oxygen concentration is not limiting with respect to radical production by TBHP. Alternatively, the

increase in the formation of methyl-DMPO adduct under anaerobic conditions might be explained by the lack of competition by O_2 with DMPO for methyl radicals. Thus, addition of methyl radicals to DMPO is more favorable under conditions of reduced oxygen concentration.

CHP has been shown to initiate lipid peroxidation in liver mitochondria; the initial step in this process is a reaction between the hydroperoxide and mitochondrial cytochrome P-450 (19). We have probed the effect of carbon monoxide, a potent inhibitor of cytochrome P-450, on TBHP-induced radical production in mitochondria to determine whether mitochondrial cytochrome P-450 is involved in the decomposition of TBHP. When mitochondria are purged with CO for 30 sec prior to the addition of TBHP, a 100% increase in the intensity of the methyl-DMPO adduct is observed (Table II). No increase is observed if the mitochondria are purged with air for 1 min following CO treatment. The fact that CO does not inhibit radical production suggests that mitochondrial cytochrome P-450 probably is not involved in the mechanism by which TBHP induces radical production in mitochondria. Further evidence for this is provided by the fact that NEM has been shown to stimulate CHP/cytochrome P-450 initiated mitochondrial lipid peroxidation (19) whereas NEM inhibits TBHP-induced radical production in mitochondria (Table II).

The effect of glutathione peroxidase, catalase and superoxide dismutase (SOD) on TBHP-induced radical production in mitochondria was studied to determine whether a role for hydrogen peroxide and superoxide exists in the mechanism of radical production. Triton X-solubilized mitochondria were used to enhance the accessibility of these enzymes to the site of interaction of TBHP. When glutathione peroxidase and GSH are added to mitochondria prior to TBHP, a 70% reduction in the intensity of the methyl-DMPO adduct is observed (Table II). This result may reflect involvement of hydrogen peroxide or it may be due to increased metabolism of the hydroperoxide since both H_2O_2 and TBHP are substrates for glutathione peroxidase (56). Addition of catalase results in a 40% inhibition of radical production (Table II). To determine if this reduction is due to destruction of H_2O_2 or is the result of a non-specific protein interaction, the mitochondria were treated with BSA prior to the addition of TBHP. The same 40% inhibition of radical production is observed with BSA (Table II), indicating that the effect of catalase is due to a non-specific protein interaction. Thus, hydrogen peroxide does not appear to be involved in hydroperoxide-induced radical production. SOD has no significant effect on the intensity of the methyl-DMPO adduct signal, suggesting that superoxide is also not involved in the

mechanism of radical production in the mitochondrial system.

Fate of Radicals in Mitochondria. We have examined the ability of TBHP to induce lipid peroxidation in rat liver mitochondria by the TBA test (28). The data in Fig. 8, Panel A shows that TBHP does induce the production of a low level of TBA-reactive materials (57). The inclusion of succinate results in a decrease in the level of TBA-reactive materials (Fig. 8, Panel A), suggesting that succinate inhibits lipid peroxidation. This result is consistent with the observation that succinate inhibits mitochondrial lipid peroxidation initiated by CHP (19). This inhibition has been attributed to the ability of succinate to reduce hydroperoxides via the glutathione peroxidase pathway (58).

To determine the role of glutathione peroxidase in the inhibition of mitochondrial lipid peroxidation, we added a glutathione peroxidase inhibitor, mercaptosuccinic acid (MSA) (59), to the mitochondria prior to the addition of succinate and TBHP. Addition of MSA results in a level of TBA-reactive materials intermediate between that seen with TBHP in the presence and absence of succinate (Fig. 8, Panel A), suggesting that MSA reduces the inhibitory ability of succinate. The fact that complete release of inhibition is not observed with MSA suggests that all of the inhibition by succinate is not due to reduction of

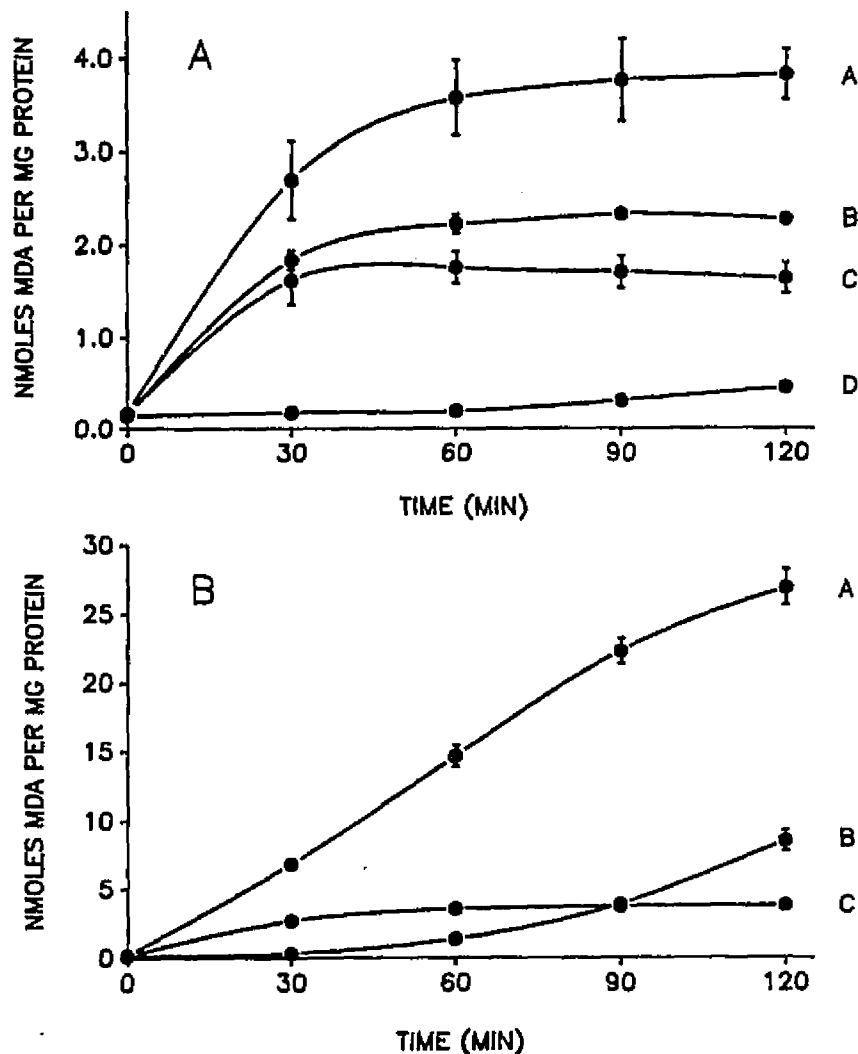


Figure 8: Effect of respiratory substrate (Panel A) and a thiol pro-oxidant (Panel B) on malondialdehyde formation in mitochondria incubated with *tert*-butyl hydroperoxide (TBHP). Panel A - (A) Incubation containing 5.0 mg mitochondrial protein and 5 mM TBHP in 0.15 M NaCl at 25°C. 1.0 ml aliquots were removed from the suspension and treated in the manner described under Methods. (B) Same as in A except 0.1 mM mercaptosuccinic acid added 3 min prior to TBHP and 15 mM sodium succinate added 1 min prior to TBHP. (C) Same as in A except 15 mM sodium succinate added 1 min prior to TBHP. (D) Same as in A except no TBHP added. Panel B - (A) Incubation containing 5.0 mg mitochondrial protein, 0.1 mM mercaptosuccinic acid and 5 mM TBHP in 0.15 M NaCl at 25°C. The mercaptosuccinic acid was added 2 min prior to the addition of TBHP. (B) Same as in A except no TBHP was added. (C) Same as in A except no mercaptosuccinic acid and TBHP were added. Each value is an average of three determinations \pm standard deviation.

hydroperoxides by the glutathione peroxidase pathway. The ESR data may provide a rationale for this difference. In the absence of respiratory substrate, TBHP induces the production of alkyl, alkoxyl and peroxy radicals. In the presence of respiratory substrate (either BHB or succinate), a significant stimulation of alkyl radical production is observed (Fig. 6) while alkoxyl radical production is apparently unchanged and no peroxy signal is present. ESR time-course studies (Fig. 9, Panel A) indicate that at the end of a 90 min period, the intensity of the alkoxyl-DMPO adduct is approximately the same in both respiring and non-respiring mitochondria while the intensity of the methyl-DMPO adduct is significantly more intense in the respiring system. These results suggest that a greater degree of lipid peroxidation in non-respiring mitochondria (Figure 8, Panel A) is due to generation of peroxy radicals in this system. An enhanced production of alkyl radicals in respiring mitochondria is expected to result in some type of mitochondrial damage since these species are known to participate in both hydrogen abstraction and addition reactions (60-62). Kensler and Taffe have suggested that methyl radicals produced in hydroperoxide-treated keratinocytes may modulate the cell phenotype during promotion or progression by alkylating cellular macromolecules (11). This is

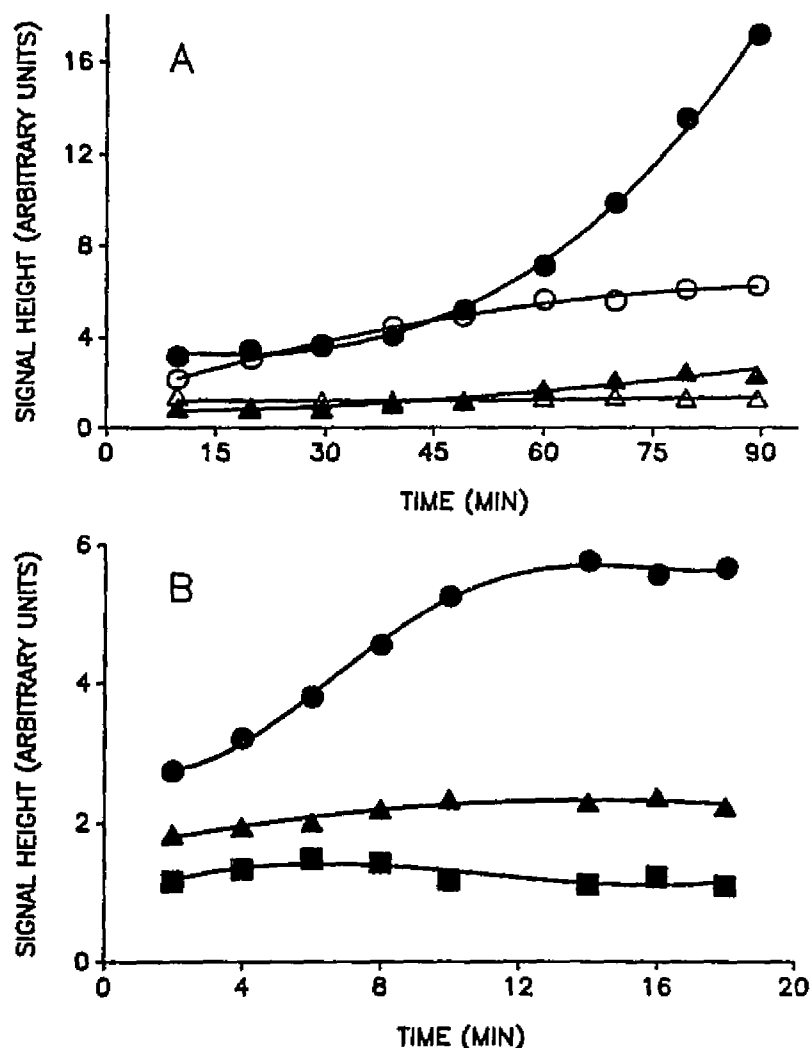


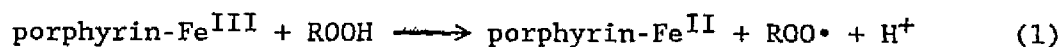
Figure 9: Change in Intensity of DMPO-Radical Adducts With Time in Mitochondria Treated With *tert*-Butyl Hydroperoxide in the Presence of DMPO. Panel A - Change in intensities of methyl-DMPO adduct (circles) and *tert*-butylalkoxyl-DMPO adduct (triangles) in respiring (closed symbols) and non-respiring (open symbols) mitochondria treated with TBHP. Incubation containing 5.0 mg mitochondrial protein, 50 mM DMPO and 1.5 mM TBHP. Substrate (10 mM sodium succinate) was added 1 min prior to TBHP in the respiring mitochondrial system. Same instrument conditions as in Fig. 1 except that single scans were recorded at 10 min intervals. Panel B - Change in intensity of methyl-DMPO adduct (circles), *tert*-butylalkoxyl-DMPO adduct (triangles) and *tert*-butylperoxyl-DMPO adduct (squares) in non-respiring mitochondria treated with TBHP. Incubation containing 5.0 mg mitochondrial protein, 50 mM DMPO and 1.5 mM TBHP. Same instrument conditions as in Fig. 1 except that single scans were recorded at 2 min intervals.

consistent with the hypothesis that methylation reactions are involved in eukaryotic gene expression (63).

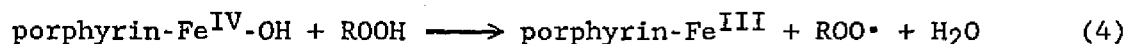
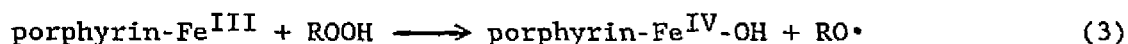
Although thiols are known to protect against lipid peroxidation (64-66), these compounds can also act as pro-oxidants (67). Thiol-dependent lipid peroxidation is suggested to depend on direct reduction of iron by the thiol (68). GSH has been shown to act as a pro-oxidant in mitochondria; the addition of this compound to mitochondria results in lipid peroxidation and swelling (69). The glutathione peroxidase inhibitor MSA is a thiol; therefore, this compound may be expected to act as a pro-oxidant in mitochondria in a manner analogous to GSH. When MSA is added to non-respiring mitochondria, a significant amount of TBA-reactive materials are formed over a period of 2 hours (Fig. 8, Panel B), indicating that this compound does induce lipid peroxidation in this organelle. A synergistic effect is observed when both MSA and TBHP are added to non-respiring mitochondria (Fig. 8, Panel B); in this system, the level of TBA-reactive materials at 120 min. is 3 times that observed with MSA alone and 7 times that observed with TBHP alone. In non-respiring mitochondria, the level of NADH and FADH₂ is low so the activity of the glutathione peroxidase pathway must also be low. In this condition, hydroperoxides will not be readily metabolized by this pathway. Thus, in a state of oxidative stress, thiols may no longer function in a protective manner but may act to

potentiate the effect of accumulated hydroperoxides, resulting in increased damage to the organelle as is observed in our in vitro system.

Mechanisms of Radical Production. Our ESR spin-trapping results indicate that TBHP interacts with a tightly-liganded metal center in non-respiring mitochondria; the site of interaction is identified as heme iron based on inhibition of radical production by sodium cyanide and lack of inhibition by metal chelators (Table II). In this regard, TBHP has been shown to react with a number of different heme proteins (32,35). In non-respiring mitochondria, electron transport chain components are in their oxidized form. Thus, in all probability the initial reaction in this system is between TBHP and heme-bound ferric iron. Heme proteins are believed to catalyze the decomposition of hydroperoxides via Fenton reactions (70,71).



An alternative mechanism has been proposed in which the initial event is homolytic cleavage of the oxygen-oxygen bond of the hydroperoxide molecule by the heme (72); this reaction results in the formation of an alkoxyl radical and heme-bound hydroxyl radical.



The products of both of these mechanisms is an alkoxyl radical, a peroxy radical and water. Several heme proteins are also believed to catalyze the decomposition of hydroperoxides via a peroxidase-type mechanism resulting in the formation of peroxy radicals (73-76).

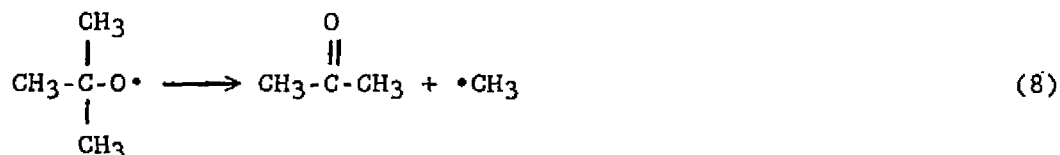


The addition of TBHP to non-respiring mitochondria in the presence of DMPO results in the formation of tert-butylperoxyl-DMPO, tert-butoxyl-DMPO, and methyl-DMPO adducts (Fig. 1). tert-Butylperoxyl radicals may be formed by either reaction 1, 4, 6 or a combination of these processes. Although tert-butoxyl radicals may result from either reaction 2 or 3, it is unlikely that reaction 2 contributes significantly in a mitochondrial system if the heme protein involved is a component of the electron transport chain. If a cytochrome is reduced by reaction 1, the extra electron would be expected to be transferred to the next component of the chain and not to the hydroperoxide. If the process in reactions 3 and 4 occurs, there is no reduction of the iron, so an electron transfer to another component of the chain would not be a

possibility in this case. Thus, the most likely reaction for the formation of tert-butoxyl radicals in the mitochondrial system is reaction 3. tert-Butoxyl radicals may also arise via a reaction between tert-butylperoxyl radicals since tertiary peroxyl radicals have been shown to undergo a self reaction (77).



Once formed, a tert-butoxyl radical may participate in an addition reaction, hydrogen abstraction reaction or may undergo beta-scission to form a methyl radical and acetone (78,79).



This is most likely the source of the methyl radicals that are observed in the form of the methyl-DMPO adduct.

Davies (32) has suggested that the peroxidase mechanism (reactions 5 and 6), which yields only peroxyl radicals, is the dominant process in a cytochrome c/TBHP system. Firstly, he observes DMPOX, which has been shown to arise via the reaction of high-valence-state (e.g., ferryl) species with DMPO (80). Secondly, the buildup of the alkoxyl-DMPO adduct appears to mirror the loss of the peroxyl-DMPO adduct. Thus, he proposes that alkoxyl-DMPO

adducts are produced by breakdown of peroxy-DMPO adducts. We do not observe DMPOX in non-respiring mitochondria treated with TBHP in the presence of DMPO, although we cannot rule out the possibility that DMPOX is formed and then reduced via endogenous reducing agents (e.g., ascorbate, GSH) in the mitochondria. We also do not observe a correlation between the loss of peroxy-DMPO adduct and formation of alkoxy-DMPO adduct (Fig. 9, Panel B); therefore, both alkoxy and peroxy radicals appear to be formed directly in the non-respiring mitochondrial system. Although the peroxidase mechanism (reactions 5 and 6) may function in this system, reactions 3 and 4 appear to be the dominant radical-producing process.

In a respiring mitochondrial system, our ESR spin-trapping results indicate that cytochrome c_1 donates electrons to reduce TBHP resulting in radical production. In the presence of DMPO, only tert-butoxy-DMPO and methyl-DMPO adducts are observed. The absence of the tert-butylperoxy-DMPO adduct suggests that TBHP only reacts with reduced heme iron in respiring mitochondria. Reaction 2 is expected to be the initial radical-producing reaction in this system, resulting in the formation of tert-butoxy radicals. These radicals then undergo beta-scission to form methyl radicals. The fact that no tert-butylperoxy radicals are trapped suggests that reaction 1 is not a contributing reaction in the respiring mitochondrial

system. This is logical since any heme iron oxidized via reaction 2 would be immediately reduced by electron transfer from the respiratory chain. Both the process shown in reactions 3 and 4 and that shown in reactions 5 and 6 require an initial reaction of the hydroperoxide with ferric iron. Thus, the contribution of these pathways should not be significant in the respiring mitochondrial system.

When CHP is added to non-respiring mitochondria in the presence of DMPO, cumylperoxyl-DMPO, cumyloxyl-DMPO and methyl-DMPO adducts are observed (Fig. 3). The relative signal intensities of the three adducts is different than those observed with TBHP; the cumylperoxyl-DMPO adduct predominates while the cumyloxyl-DMPO adduct is almost non-existent. This difference may suggest a change in mechanism of radical production. It may be that in the case of CHP, the peroxidase mechanism (reactions 5 and 6) is dominant. This also is reasonable since CHP is known to interact with mitochondrial cytochrome P450 to yield peroxyl radicals (19).

Summary. TBHP induces the production of alkoxyl, methyl and peroxyl radicals in non-respiring rat liver mitochondria as shown by ESR spin-trapping results. Studies with a number of additives suggest that TBHP interacts with a heme iron-containing protein to initiate radical production. A significant increase in methyl

radical production is observed in respiring mitochondria; the magnitude of increase has been shown to be related to the respiratory control ratio of the mitochondrial preparation. Our studies indicate that cytochrome c_1 donates electrons to the hydroperoxide in this system. CHP and LHP have also been shown to induce radical production in liver mitochondria.

TBHP and CHP have been shown to induce radical production in keratinocytes via interaction with a cytosolic species (15), no radical production is observed with the mitochondrial fraction (15). If the tumor-promoting ability of peroxides requires radical production, then peroxide-mitochondrial interactions may not be important in skin tumor promotion. However, in liver, peroxide-mitochondrial interactions may be important in peroxide-mediated tumor promotion since hydroperoxides induce radical production in this organelle.

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VITA

Christopher Henry Kennedy was born on November 13, 1961 in Corry, Pennsylvania. He is the son of John R. and Patricia J. Kennedy of Fairview, Pennsylvania. He graduated from Fairview High School in 1979. He earned his B.S. in Chemistry from Edinboro University of Pennsylvania in 1983. He is a member of the American Chemical Society, Phi Lambda Upsilon Honorary Chemical Society, Alpha Chi Honor Fraternity and Phi Mu Alpha Sinfonia Professional Music Fraternity. He is currently a Ph.D. candidate in the Chemistry Department of Louisiana State University. He has received a NIH National Research Service Award (5T32 ES-07126) to pursue postdoctoral research at the National Institute of Environmental Health Sciences in Research Triangle Park, North Carolina.

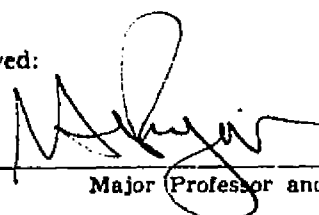
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Christopher Henry Kennedy

Major Field: (Organic) Chemistry

Title of Dissertation: A Study of the Interaction of Peroxidic Tumor Promoters
With Mitochondria

Approved:

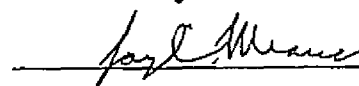
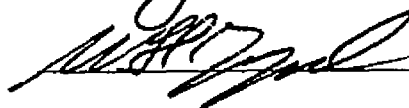
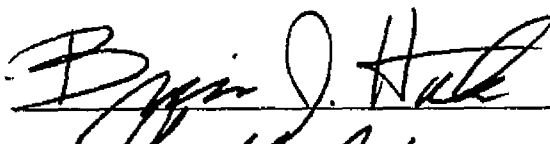


Major Professor and Chairman



Dean of the Graduate School

EXAMINING COMMITTEE:



Date of Examination:

December 2, 1988